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Exploring the effects of Cadherin-derived Peptides on Platelet and Endothelial Cell Function

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Publications

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Conferences

International

32nd European Peptide Symposium, Athens, September 2012 – *Identification of Novel anti thrombotic Cadherin-derived peptides* (Poster)

UK Platelet Conference, Cardiff, July 2011 – *Characterization of Cadherin derived peptides that inhibit secretion in Human Platelets* (Poster)

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Irish Platelet Meeting, UCD, November 2011 – *Characterization of Cadherin derived peptides that inhibit secretion in Human Platelets* (Poster)

Internal

RCSI Research Day, 2013 – *Discovery VE-Cadherin in Human Platelets* (Poster)

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Abstract

The primary role of platelets is hemostasis, the prevention of excessive bleeding following vascular injury. However, platelets also play a role in the pathology of cardiovascular disease. Platelet mediated thrombosis on ruptured atherosclerotic plaques underlies the acute complications of cardiovascular disease including myocardial infarction and ischemic stroke. Arterial thrombus formation is a complex process involving various cell adhesion molecules and secondary mediators, such as ADP, secreted from activated platelets, which help to recruit platelets. There is an ongoing search for anti-platelet agents that can prevent thrombotic events whilst minimizing bleeding.

Previous work in our laboratory identified a palmitoylated peptide derived from Kidney Cadherin (K-Cadherin) as a significant inhibitor of platelet function. Based on this observation, the anti-platelet effects of other cadherin-derived peptides were investigated. Firstly, the effect of peptides derived from membrane-adjacent cytoplasmic regions of K-, Epithelial Cadherin (E-Cadherin) and Neural Cadherin (N-Cadherin) sequences were assessed in assays of platelet function. Although many of these peptides were shown to inhibit platelet secretion and aggregation responses, it emerged that the corresponding control peptide sequences showed a similar ability to inhibit platelet function. Thus it seemed that many of the observed effects were non-specific.

Secondly, I identified a novel junctional protein, Vascular Endothelial Cadherin (VE-Cadherin) and its associated protein, P120-catenin in human platelets and demonstrated, for the first time, that VE-Cadherin could form a heterophilic interaction with platelet integrin αIIbβ3. This interaction was Arginine-Glycine-Aspartic acid (RGD) dependent. Peptides derived from juxtamembrane domain (JMD) of VE-Cadherin, such as those studied in the first results chapter, had only non-specific effects on platelet function.

Thirdly, the effect of the VE-Cadherin-derived peptides on endothelial cell function and angiogenesis were investigated. Non-specific activity was carefully monitored using matched control peptides and lower peptide doses than in previous studies. This study demonstrates that certain VE-Cadherin peptides specifically inhibited endothelial cell functions, including angiogenesis, migration and proliferation. These results highlight an important role played by the JMD in VE-Cadherin and identified potential inhibitors of cadherin function that may have therapeutic relevance.

In the final chapter of this thesis, I investigated which parameters of cadherin-derived peptides, and their control peptides, were associated with the anti-platelet effect. It was found that water-soluble peptides with positively charged amino acids at their N-terminus are associated with anti-platelet activity. In addition, this prediction was also experimentally verified by using peptides with positively charged amino acids.

Taken together, this thesis explores the cellular effects of cell-permeable peptides derived from target proteins. It clearly identifies a need for appropriate control peptides in all such experiments to control for sequence, dose and toxicity. Within these constrains, novel peptides were identified that inhibit VE-Cadherin function in endothelial cells.

Abbreviations

AA	Amino acid
ACD	Acid citrate dextrose
ADP	Adenosine diphosphate
AJ	Adherens junctions
AP2	Adapter protein 2
APS	Ammonium persulfate
ARM	Armadillo repeat proteins
ATP	Adenosine triphosphate
AU	Arbitrary units
AUP1	Ancient ubiquitous protein 1
BSA	Bovine serum albumin
CaCl2	Calcium chloride
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
CBB	Coomassie brilliant blue
CBD	Catenin binding domain
CEA	Carcinoembryonic antigen
CHO	Chinese hamster ovary cells
CIB1	Calcium and integrin binding 1
CID	Collision induced dissociation
Csk	C-terminal Src kinase
CXCR4	Chemokine CXC-type receptor 4
DAG	Diacylglycerol
DEP1	Density enhanced protein-1

DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DTS	Dense tubular system
DTT	Dithiothreitol
E-Cadherin	Epithelial Cadherin
EC	Extracellular domains
ECM	Extracellular matrix
EDC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
EGF	Endothelial growth factor
EPGF	Epidermal growth factor
FAK	Focal-adhesion kinase
FBS	Fetal bovine calf serum
FcRγ	Fc receptor γ-chain
FGF	Fibroblast growth factor
FoxO1	Forkhead-box protein-O1
FPR2	Formyl peptide receptor-2
FRET	Fluorescence resonance energy transfer
GP	Glycoprotein
GPCR	G-protein coupled receptors
GPM	Global proteome machine
GPS	Gray platelet syndrome
GTP	Guanosine triphosphate
HPS	Hermansky-Pudlak Syndrome
HPLC	High performance liquid chromatography

HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cells
ICln	Chloride ion current inducer protein
IGF	Insulin-like growth factor
IL	Interleukin
IP_3	Inositol trisphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
ITG	Integrin
JAM	Junctional adhesion molecule
JMD	Juxtamembrane domain
K-Cadherin	Kidney Cadherin
KGD	Lysine-Glycine-Aspartic acid
LAT	T lymphocyte adapter protein
LCMS	Liquid chromatography–mass spectrometry
LDH	Lactate dehydrogenase
LRR	Leucine rich repeats
MC4	Melanocortin 4
MGF	Mascot generic file format
MS	Mass spectrometry
N-Cadherin	Neural Cadherin
NAD	Nicotinamide adenine dinucleotide
NP40	Nonyl phenoxypolyethoxylethanol 40
OCS	Open canalicular system
PAGE	Polyacrylamide gel electrophoresis
Pal	Palmitoylated

PAR	Protease activated receptor
PBS	Phosphate buffer saline
PDGFR	Platelet-derived growth factor receptor
PECAM	Platelet endothelial cell adhesion molecule
PFA	Paraformaldehyde
PGE_1	Prostaglandin E_1
PGE_2	Prostaglandin E_2
PI3K	Phosphatidylinositol-3-OH-kinase
PKC	Protein kinase C
PLCγ2	Phospholipase Cγ2
PMSF	Phenylmethylsulfonyl fluoride
PNPP	Para-nitrophenyl phosphate
PP1C	Protein phosphatase 1C
PRP	Platelet rich plasma
PSG	Pregnancy specific glycoprotein
PVDF	Polyvinylidene difluoride
RBC	Red blood cell
RGD	Arginine-Glycine-Aspartic acid
RLC	Relative local conservation
RN181	Ring finger protein 181
RPM	Revolutions per minute
S1P3	Sphingosine-1-phosphate
SDS	Sodium dodecyl sulfate
SEM	Standard error mean
siRNA	Small interfering ribonucleic acid

SLIM	Short linear motif
SLP-76	Lymphocyte cytosolic protein 2
TEMED	Tetramethylenediamine
TFA	Trifluoroacetic acid
TGFβ	Transforming growth factor-β
TM	Transmembrane
TIM	Triosephosphate isomerase
TP	Thromboxane receptors
TRAP	Thrombin receptor activating peptide
Tris HCL	Tris hydrochloric acid
Ttds	(1-amino-4,7,10-trioxa-13-tridecanaminesuccinimic acid)
TXA_2	Thromboxane A_2
VE-Cadherin	Vascular Endothelial Cadherin
VE-PTP	Vascular endothelial protein Tyr phosphatase
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth receptor-2
VWF	Von Willebrand factor
WP	Washed platelets

Chapter 1

General Introduction

1.1 Hemostasis

Hemostasis is a complex process, which plays a major role in the prevention of blood loss during damage to blood vessels. This process is highly conserved from zebra fish to humans (Versteeg et al., 2013). Platelets are small anucleate blood cells, which play major role in hemostasis. In the normal blood circulation, the endothelium presents a non-adhesive surface to the platelets. Following damage to the blood vessel, platelets become exposed to prothrombotic sub-endothelial matrix proteins or to soluble platelet agonists. This process triggers platelet activation and contributes to thrombus formation around the damaged area to stop blood loss. In addition, under certain pathological conditions platelets form a clot in intact blood vessels, which underlies a number of cardiovascular diseases such as atherosclerosis and myocardial infarction (Lievens and von Hundelshausen, 2011). Platelets also play a role in biological processes other than hemostasis, which includes inflammation, angiogenesis, wound healing and more recently, there is a major focus on the role of platelets in cancer metastasis (Smyth et al., 2009, Patzelt and Langer, 2012, Gay and Felding-Habermann, 2011, Mitrugno A, 2013).

1.2 Platelet structure

The platelet plasma membrane is relatively smooth in comparison to circulating leukocytes except for tiny folds on the membrane, which resemble gyri and sulci on the surface of the brain (White and Escolar,

1993). Platelets exhibit an open canalicular system (OCS) that acts as the pathway for transport of substances in and out of the cell (Escolar and White, 1991). An internal smooth endoplasmic reticulum membrane system in platelets, called the dense tubular system (DTS), plays a pivotal role in the initiation and modulation of platelet activation. (Gerrard et al., 1978). The three major platelet cytoskeleton components are the spectrin membrane skeleton, the marginal microtubule coil and the actin cytoskeleton (Smyth et al., 2009). The platelet cytoplasm contains α-granules, dense granules, lysosomes, glycosomes and mitochondria.

Alpha-granules are the most numerous platelet organelles and their number depends on the size of the platelets (Smyth et al., 2009). The α-granule contents are released during platelet activation at the damaged vessel walls and thus play an important role in hemostasis, inflammation (Harrison and Cramer, 1993) and angiogenesis (Italiano et al., 2008). Platelet dense granules are smaller and fewer in number than α-granules. Dense granules composed of adenine nucleotides, which they release upon platelet activation (Smyth et al., 2009). Platelets contain a small number of lysosomes, which are produced from the endosomal membrane system(Smyth et al., 2009).

1.3 Platelet activation

Due to their small size, platelets are pushed to the periphery of the blood vessels (Michelson, 2007). This effect enhances their ability to detect and respond to vascular damage. However, most of the platelets never

undergo significant interaction with the endothelial cell surface within the intact blood vessel (Michelson, 2007). Only at sites of vascular injury, where platelets are exposed to the subendothelial extracellular matrix, do platelets adhere, limiting hemorrhage and promoting tissue healing. Platelet activation is characterised by several processes including adhesion, shape change, secretion and aggregation. These processes are described in more detail below.

1.3.1 Platelet adhesion

At the site of vascular injury, platelet adhesion is a key process in initiating hemostasis under shear conditions. Platelet adhesion involves an interaction between platelet cell adhesion molecule, glycoprotein (GP) Ibα and the soluble plasma protein von Willebrand factor (VWF) (Varga-Szabo et al., 2008). This initial interaction allows platelets to bind to exposed collagen, a sub-endothelial matrix protein. Binding to collagen triggers intracellular signaling cascades that lead to platelet activation and firm adhesion. In addition to GPIbα, platelets express several other adhesion receptors including GPVI and integrins, in particular the platelet specific integrin αIIbβ3 (Michelson, 2007). The interaction between adhesion receptors and soluble ligands is dependent on platelet activation status, shear rate of blood flowing in the blood vessel (Savage et al., 1998) and the availability of the ligands.

1.3.2 Platelet secretion

The prominent structural features of a platelet are their three granules namely dense granules, α–granules, and lysosomes. Exposure of platelets to prothrombotic ligands (e.g. collagen and thrombin) causes platelet activation and the release of the granular contents. Hermansky-Pudlak Syndrome (HPS) is a pathological condition of platelets which results from a deficiency in dense granules (White, 1986, Swank et al., 1998) and Gray Platelet Syndrome (GPS) a deficiency of α-granules (Smith et al., 1997, Nurden and Nurden, 2007). Recent studies have shown that cystic fibrosis patients display low levels of dense granule secretion compared to normal individuals (McGivern et al., 2013). Platelet dense granules contain components such as adenosine diphosphate (ADP), adenosine triphosphate (ATP), and serotonin as well as calcium. Their release is necessary to trigger further platelet activation (platelet recruitment) and vasoconstriction (Smyth et al., 2009).

Alpha-granules are composed of a wide array of proteins that play a role in several pathological conditions. Of particular importance are adhesive proteins such as fibrinogen, fibronectin, vitronectin and thrombospondin, which are released from α-granules and play a key role in platelet-platelet binding and subsequent clot formation. Other membrane proteins in α-granules, such as P-selectin, are externalized to the plasma membrane upon platelet activation (Smyth et al., 2009). Platelets also secrete a wide range of proteins such as coagulation proteins (Factor XIII and factor V) and multiple cytokine-like proteins such as insulin-like growth factor

(IGF)-1, vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). These proteins are known to promote wound healing, inflammation and angiogenesis (Italiano et al., 2008). The wide variety of proteins and molecules in the platelet relesate suggest that platelet secretion is a key process in establishing the microenvironment around the damaged site. Therefore, control of granule release may be an effective strategy to manipulate the wound microenvironment. Recent studies have shown that platelets exhibit heterogeneity in their α-granule secretion. In humans, platelets majorly secrete at least two classes of granules depending on the nature of the agonist (Italiano et al., 2008, Ma et al., 2005). Storrie and colleagues initially reported that major adhesive proteins including fibrinogen and VWF packed differently in platelets α-granule proteins. This supports the differential release of α-granule proteins during platelet activation (Sehgal and Storrie, 2007). The differential release of α-granules is of great interest, since agents that can manipulate this release might have potential roles in the development of novel platelet function modifiers.

Platelet lysosomes contain approximately 13 acid hydrolases. The process by which lysosomes secrete their contents is uncertain, but it is known that they require much stronger stimulus than α and dense granules. Release of lysosomes is an indication of high platelet activation (Smyth et al., 2009).

1.3.3 Platelet shape change

Platelet shape change is the first event that is observed in platelet activation and it occurs in response to various agonists. The initial noticeable change following platelet activation is the breakdown of microtubule and conversion of platelets from a disc-like to sphere-like shape. Filopodia and lamellipodia, generated by new actin filament assembly, then extend from the plasma membrane. In parallel, intracellular organelles and the dismantled microtubule coil are compressed into the centre of the platelet. There are number of proteins involved in the control of platelet shape change (e.g. actin, profilin, gelsolin and caldemson) (Smyth et al., 2009) .

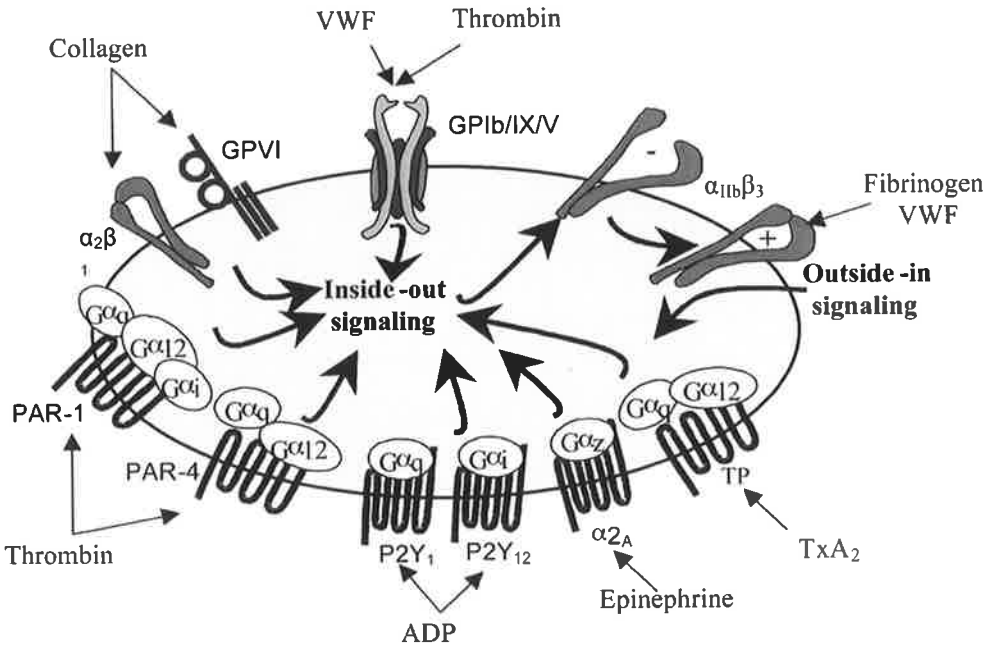
1.3.4 Platelet aggregation

Platelet aggregation is a process in which platelets adhere to each other at sites of vascular injury to prevent excessive blood loss. Platelet aggregation involves several ligands such as fibrinogen, fibronectin and VWF and receptors such as GPIbα and integrin αIIbβ3 (Jackson, 2007, Munnix et al., 2009, Brass et al., 2005). Integrin αIIbβ3 is a platelet specific receptor that is involved in platelet aggregation and is also known as GPIIb-IIIa. Integrin αIIbβ3 is the major platelet integrin, with 50,000 to 80,000 copies on each platelet (Wagner et al., 1996). Integrin αIIbβ3 has a low affinity for its ligand, plasma fibrinogen, when platelets are in their resting condition. However, its affinity dramatically increases upon platelet activation (Ma et al., 2007). Aggregation is the final step of

platelet activation, caused by the binding and cross-linking of fibrinogen by αIIbβ3 on adjacent platelets.

1.4 Platelet signaling

Hemostatic and pathological functions of platelets require a co-ordinated series of events, involving platelet membrane receptors, bidirectional intracellular signals, release of platelet proteins and inflammatory substances. To facilitate this, platelets express a wide variety of transmembrane receptors. The interaction between platelet receptors and their ligands in platelet hemostatic function is well established (Figure 1) and these receptors are briefly summarized in the next section.



Rivera J et al. Haematologica 2009;94:700-711

Figure 1 Major platelet receptors and ligand interactions. Protease active receptors (PARs), Thromboxane receptors (TP), Von Willebrand factor (VWF) and Glycoprotein (GP) receptors are indicated in this diagram along with their activating ligands.

1.4.1 Protease activated receptors (PARs)

Thrombin is a potent platelet agonist generated by the coagulation cascade of plasma proteins in response to vessel injury. Its activity is primarily mediated by PAR1 and PAR4 receptors in platelets. PAR receptors are a subfamily of G-protein coupled receptors (GPCRs). Activation of these receptors triggers platelet secretion and aggregation (Kahn et al., 1999, Coughlin, 2005). Thrombin is an enzyme, which cleaves the externally facing amino-terminal of PARs and unmasks the new amino-terminal tethered ligand that binds intramolecularly to the body of the receptor to induce transmembrane signaling in platelets (Coughlin, 2000). The tethered ligands of PARs are also referred as PAR activating peptides. The sequences of these peptide ligands are SFLLRN for PAR1 (Vu et al., 1991) and GYPGKF for PAR4 (Faruqi et al., 2000). The SFLLRN peptide from PAR1 is also called thrombin receptor activating peptide (TRAP). PAR1 can mediate platelet activation at low concentrations of thrombin but PAR4 mediates human platelet activation only at high concentrations due to its low affinity for thrombin (Kahn et al., 1999).

In human platelets both PAR1 and PAR4 will induce $G_{\alpha 12/13}$ and $G_{\alpha q}$ signaling cascades respectively (Offermanns, 2006). $G_{\alpha 12/13}$-mediated calcium/calmodulin and Rho/Rho-kinase signaling pathways are involved in initial reorganization of the platelet cytoskeleton (shape change) (Bauer et al., 1999), whereas $G_{\alpha q}$ signaling is required for platelet granule secretion (Offermanns et al., 1997). $G_{\alpha q}$ activates phospholipase C and

its downstream signaling molecules, diacylglycerol (DAG) and inositol trisphosphate (IP_3) to enable calcium release from platelet stores. Recent studies have shown that PAR1 signaling desensitization is counteracted by PAR4 signaling in human platelets (Falker et al., 2011). Due to the key role of PAR1 in platelet function, it has been targeted with compounds such as vorapaxar and atopaxar to treat cardiovascular diseases (Tello-Montoliu et al., 2011).

1.4.2 Thromboxane A_2 (TXA_2) receptors

Elevation of intracellular calcium levels by various platelet agonists stimulates release of the membrane phospholipid, arachidonic acid, and its subsequent metabolism to TXA_2 (Smyth et al., 2009). TXA_2 is a potent platelet agonist that causes secretion, shape change and aggregation (Hamberg et al., 1975). Platelets express two types of TXA_2 receptors encoded from same gene ($TXA_2\alpha$ and $TXA_2\beta$) (Hirata et al., 1996). Thromboxane receptors have been shown to interact with G-proteins to induce or augment platelet activation (Hourani and Cusack, 1991, Shen and Tai, 1998).

1.4.3 Adenosine diphosphate (ADP) receptors

ADP is a one of the key platelet agonists and plays a vital role in hemostasis. Platelets secrete ADP from dense granules upon their activation. P2Y1, P2Y12 and P2X1 are the ADP receptors present on platelets and these receptors are involved in various aspects of ADP

induced platelet activation such as shape change, secretion, aggregation and extracellular calcium influx (Murugappa and Kunapuli, 2006). All the ADP receptors are GPCRs. P2Y1 activates platelets via a $G_{\alpha q}$ /phospholipase C pathway leading to generation of second messengers like IP_3 and diacylglycerol (DAG) (Offermanns et al., 1997). IP_3 mobilizes intracellular calcium stores while DAG activates the protein kinase C isoforms, PKCα and β (Heemskerk et al., 2011). This activation leads to shape change and ADP mediated platelet aggregation and thromboxane generation (Jin et al., 2002). The P2Y12 receptor induces activation signals, also through G-proteins, that inhibit the elevation of cyclic adenosine monophosphate (cAMP) levels mediated by prostaglandin I_2 (PGI_2) (Yang et al., 2002).

1.4.4 Glycoprotein (GP) VI

GPVI is a major collagen receptor on platelets and belongs to the immunoglobulin superfamily (Clemetson and Clemetson, 2001). It plays a major role in recruiting platelets to the site of vascular injury. Deficiency of GPVI leads to decreased collagen-dependent platelet adhesion and aggregation and is associated with a bleeding disorder (Moroi et al., 1989). GPVI is constitutively associated with Fc receptor γ-chain (FcRγ), a protein that contains an immunoreceptor tyrosine-based activation motif (ITAM) (Gibbins et al., 1997, Berlanga et al., 2002). GPVI associates with FcRγ chain through a salt-bridge between charged and basic amino acids (AAs) within the short linear transmembrane motif and through specific sequences within the proximal juxtamembrane of GPVI (Bori-Sanz et al.,

2003). Binding of collagen to GPVI causes Src-kinase dependent phosphorylation of the FcRγ ITAM leading to activation of Syk. The Src family kinases Lyn and Fyn, play a crucial role in this initial process (Ezumi et al., 1998). Initiation of this step leads to formation of a large signaling complex. The major signaling molecule in this complex is phospholipase Cγ2 (PLCγ2). Activation of this complex signaling cascade leads to liberation of secondary messengers such as DAG and IP_3 (Watson et al., 2005). Adaptor proteins such as, Lymphocyte cytosolic protein 2 (SLP-76) and T lymphocyte adaptor protein (LAT) regulate the activation of PLCγ2 (Judd et al., 2002).

1.4.5 Integrins

Integrins are the major receptors for cell adhesion to the extracellular matrix (Humphries, 2000). They consist of heterodimers of α and β subunits associated in 1:1 ratio. Both α and β subunits are non-covalently associated, type I transmembrane proteins with large extracellular domains and short cytoplasmic tails (Humphries, 2000). To date, eighteen α subunits and eight β subunits have been identified forming twenty-four heterodimers. In addition to adhesion, integrins bind to the intracellular cytoskeleton and transduce many intracellular signaling pathways (Hynes, 2002). Platelets express six different types of integrins αvβ3, α2β1, α5β1, α6β1, αLβ2 and αIIbβ3.

Integrin αIIbβ3 is a platelet specific integrin (Wagner et al., 1996). It is also the most abundant of all the platelet integrins accounting for >50,000

copies per platelet (Wagner et al., 1996). It has been calculated that this integrin alone covers between 12 and 20% of the total platelet surface (Moran et al, 2000). Upon endothelial damage, platelets form a plug around the wound area by cross-linking of platelets via αIIbβ3 and the soluble plasma protein fibrinogen. Integrin αIIbβ3 has several ligands such as fibrinogen, VWF, fibronectin, vitronectin and CD40L (Marguerie et al., 1979, Timmons et al., 1984, Plow et al., 1985). A recent study has shown that K-Cadherin is also a novel ligand of platelet integrin αIIbβ3 (Dunne et al., 2012). However, it is unclear from the Dunne *et al.* study whether K-Cadherin binds to resting or activated integrin αIIbβ3 (Dunne et al., 2012). Deficiency of αIIbβ3 on platelets is associated with a pathological bleeding condition called Glanzmann's thrombasthenia (Phillips and Agin, 1977).

On circulating platelets, αIIbβ3 is present in a resting and low affinity conformation (Ma et al., 2007). Stimulation of platelets with soluble agonists such as ADP, TXA_2 and thrombin induces transformation of αIIbβ3 to higher affinity conformation. This phenomena is referred to as *Inside-out signaling* (Ma et al., 2007). This transition is very rapid and depends on a series of signaling events from several receptors that culminate in the binding of cytoskeletal proteins, including talin and kindlin-3, to the cytoplasmic tail of the β3 subunit (Shattil et al., 2010, Ma et al., 2007). Binding of fibrinogen to activated integrin also contributes to platelet activation (Buensuceso et al., 2004). This is called *Outside-in signaling*. Most of the integrin ligands, such as fibrinogen, contain a

common sequence or motif, arginine-glycine-aspartic acid (RGD). RGD-containing peptides bind to the extracellular domain of several integrins and can inhibit the binding of other ligands (fibrinogen, VWF, fibronectin, vitronectin and CD40L) and block platelet aggregation (Bledzka et al., 2013). RGD peptides were used as a starting point in the design of αIIbβ3 antagonists like Tirofiban (Egbertson et al., 1994). Due to the key role of αIIbβ3 in platelet function, it was identified as major target to develop anti-thrombotic drugs such as Abciximab and Integrilin (cyclic Lysine-Glycine-Aspartic acid (KGD)) (Bledzka et al., 2013). In addition to this, a number of studies have targeted the activity of αIIbβ3 using cell permeable peptides (Koloka et al., 2008, Dimitriou et al., 2009, Stephens et al., 1998).

A number of proteins have been shown to bind to the cytoplasmic domains of αIIb and/or β3, either directly or through interactions with other proteins. The integrin αIIb binding proteins include, calcium and integrin binding 1 (CIB1), ancient ubiquitous protein 1 (AUP1) 189, protein phosphatase 1C (PP1C), triosephosphate isomerase (TIM), chloride ion current inducer protein (ICln), and ring finger protein 181 (RN181) (Bennett, 2005, Liu et al., 2000, Larkin et al., 2004). Integrin β3 subunit binding proteins are talin, filamin, paxillin, kindlin-3 and focal adhesion kinase (FAK) (Bennett, 2005, Liu et al., 2000). Binding of these proteins to integrin subunits regulates further downstream signaling events such as platelet cytoskeletal rearrangement and granule secretion (Smyth et al., 2009). A greater understanding of key regions in αIIbβ3 and

its regulated proteins will aid in the development of novel anti-thrombotic drugs.

1.5 Cadherins

Cell-cell adhesion is a complex system in both structural and functional aspects and requires an understanding of a number of biochemical concepts including tight junctions, adherens junctions, gap junctions and desmosomes (Takeichi, 1988). Adherens junctions (AJ) are important components of cell-cell interactions, which contribute to events such as embryogenesis and tissue morphogenesis (Harris and Tepass, 2010). Cadherins are the core components of AJ (Harris and Tepass, 2010) and form calcium-dependent homophilic interactions with neighbouring cells at AJs (Takeichi, 1988).

Most cadherins are single-pass transmembrane proteins found on cell membranes at points of cell-cell contact. The cadherin super family is divided into different subfamilies based on protein domain composition, genomic structure, and phylogenetic analysis of the protein sequences. Different families of cadherins include, classical type-I, type-II cadherins, desmosomes, protocadherins, Fat-like family, Dachsous and Flamingo cadherins (Figure 1.2) (Nollet et al., 2000).

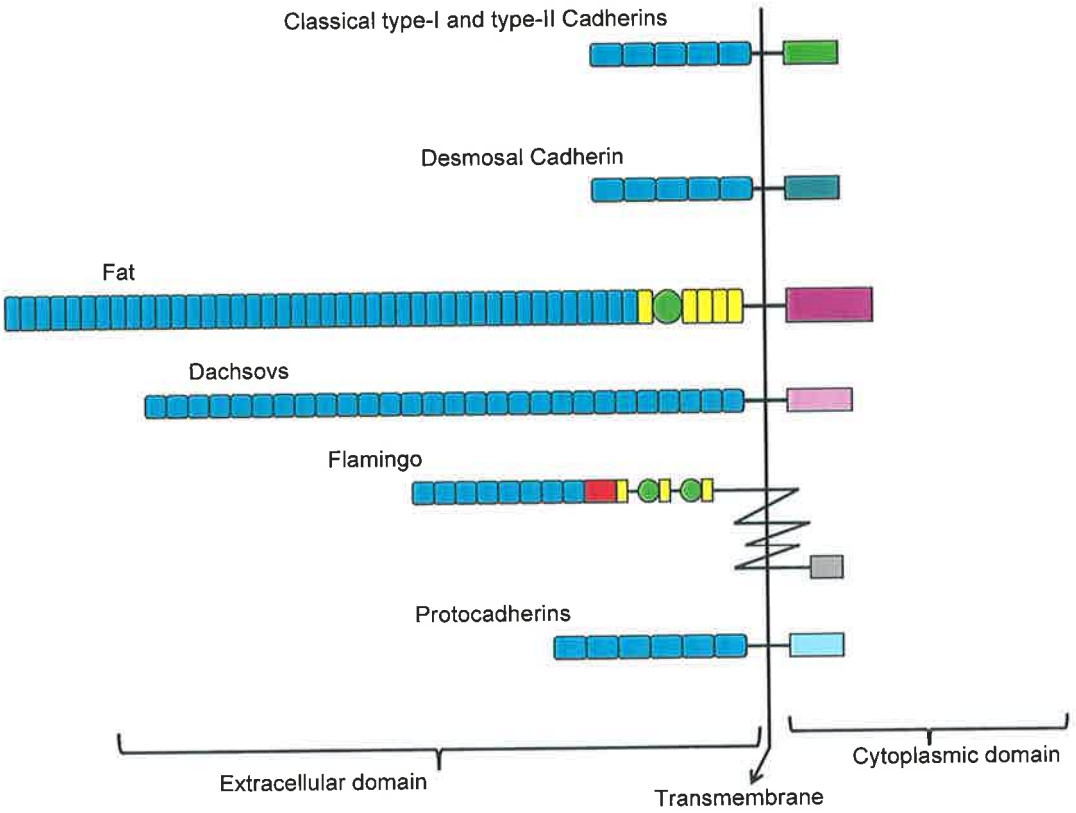


Figure 1.2 Schematic representation of cadherin superfamily. Most of the cadherins are single pass transmembrane proteins except Flamingo cadherins. Flamingo cadherins are 7 transmembrane proteins. Fat cadherin is the largest cadherin, with 34 cadherin repeats. Blue ovals and green circles indicate cadherin repeats and laminin A-G motifs, respectively. Yellow boxes indicate epidermal growth factor (EPGF) motifs. The red box in Flamingo indicates the flamingo box. The variously colored rectangles indicate the intracellular domains of cadherins. This figure was generated based on information from (Nollet et al., 2000).

Most common cadherins are classical type-I and type-II cadherins (Table 1.1). These cadherins consists of five extracellular domains (EC) followed by a transmembrane domain and one cytoplasmic domain (Figure 1.2) (Nollet et al., 2000). The EC of classical cadherins are highly homologous domains known as cadherin domains (Ivanov et al., 2001). Although a membrane linked extracellular domain of a cadherin protein is sufficient for calcium dependent homophilic interactions, it is well known that the interaction of the cytoplasmic tail with the cytoskeleton, along with

receptor clustering, significantly increases the strength of cadherins at Adherens junctions (AJs) (Yap et al., 1997). The conformation of the cadherin molecule is stable only in the presence of Ca^{2+}. The calcium-binding sites in cadherins, consist of highly conserved amino acid (AA) short sequences located between adjacent extracellular repeats (Ivanov et al., 2001). The highly conserved cytoplasmic domain of classical cadherins binds to cytoplasmic proteins known as catenins namely P120-catenin and β-catenin. Beta-catenin binds to α-catenin and form a cadherin-catenin complex (Harris and Tepass, 2010). Catenins are Armadillo repeat proteins (ARM) which bind to the cytoplasmic tails of cadherins to support their stability and mediate cadherin cytoplasmic functions (Shapiro and Weis, 2009). The membrane proximal, cytoplasmic region of cadherins is known as the juxtamembrane domain (JMD). The JMD of cadherins binds to P120-catenin and regulates cadherin endocytosis (Daniel and Reynolds, 1995, Thoreson et al., 2000, Davis et al., 2003). The JMD usually consists of 40–80 residues and contains several basic amino acids (AAs) (Lysine and Arginine) located close to the transmembrane region.

The exact binding mechanism of five different extracellular domains of cadherins is still unknown. However, the interactions between extracellular domain (EC) 1 and EC2 domains in type-I and type-II cadherin-cadherin interactions have been well studied. Both type-I and type-II cadherins bind via a swapping mechanism involving their N-termini. This mechanism involves binding of the N-terminus of cadherin

EC1 between opposing cells across the intracellular space by a reciprocal exchange of N-terminus β-strands to form standard swapped dimers (Patel et al., 2006). This standard exchange principle is similar in both type-I and type-II cadherins but it differs at molecular level (Patel et al., 2006). Several residues in the extracellular domain (EC) 1 of cadherins are involved in homophilic binding. The type-I cadherin dimer structure reveals that conserved Tryptophan 2 (W2) binds to a hydrophobic acceptor pocket in the partner EC1 domain, anchoring the swapped dimer (Patel et al., 2006, Brasch et al., 2011). In type-II cadherins, two anchoring tryptophan residues were observed. W2 and W4, which are bind in an acceptor pocket in adjacent cadherin that is larger in type-II compared to type-I (Brasch et al., 2011, Patel et al., 2006). Figure 1.3 shows the graphical representation of VE-Cadherin (example of Type II cadherins) homophilic interaction in its first extracellular domain.

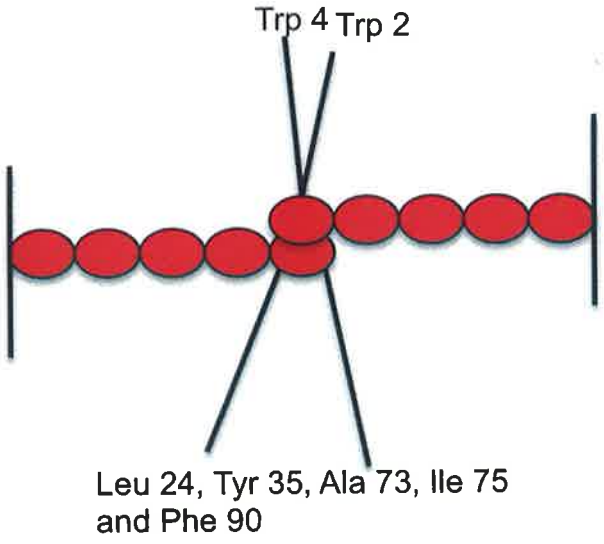


Figure 1.3 Graphical representation of potential residues involved in VE-Cadherin (example of type-II cadherin) homophillic binding. Trp 2 and Trp 4 from the first extracellular domain (EC) of VE-Cadherin binds to a hydrophobic acceptor pocket in the extracellular domain 1 of other VE-Cadherin.

Cadherins are involved in many biological processes such as embryonic development, regulation of apoptosis, tissue morphology and cell differentiation (Vleminckx and Kemler, 1999). Cadherins are also implicated in the cellular events involved in cancer. During primary tumour development, cells often have abnormal cadherin expression (Berx and van Roy, 2009). This property is important during development when single cells need to join to one another and form a coherent tumour. In addition, tumour progression correlates with loss of overall cadherin expression or loss of cadherin dependent cell–cell contacts (Berx and van Roy, 2009). This loss of cadherin normal localization causes dysregulation of cell-cell adhesion, which helps to enhance cell motility. Removal of contact suppression of growth results in uncontrolled proliferation and invasion of tumour cells (Berx and van Roy, 2009).

Table 1.1 List of classical type-I and type-II cadherins and their Acronyms (acronyms were obtained from www.uniprot.org). This list was generated from (Nollet et al., 2000).

Name	Acronym
Classica/ type-I Cadherins	
Cadherin-1	E-Cadherin
EP-Cadherin	C-Cadherin
Cadherin-2	N-Cadherin
Cadherin-3	P-Cadherin
Cadherin-4	R-Cadherin
Cadherin-15	M-Cadherin
Cadherin-13	H-Cadherin
A typical /type II Cadherins	
Cadherin-5	VE-Cadherin
Cadherin-6	K-Cadherin
Cadherin-7	CDH7L1
Cadherin-8	None
Cadherin-9	None
Cadherin-10	T2-Cadherin
Cadherin-11	OB-Cadherin
Cadherin-12	BR-Cadherin
Cadherin-18	Cadherin-14
Cadherin-19	CDH7L2
Cadherin-20	CDH7L3

1.5.1 Vascular endothelial cadherin (VE-Cadherin)

VE-Cadherin belongs to the type-II classical cadherin family discovered by Suzuki *et al.* (Suzuki et al., 1991). Sequence comparison analysis identified a number of structural features of VE-Cadherin that are shared with other cadherin family members (Lampugnani et al., 1992, Tanihara et al., 1994).

The importance of VE-Cadherin has been studied using various approaches. VE-Cadherin is abundantly expressed in vascular endothelial cells. A gene deletion study identified that VE-Cadherin is crucial for embryonic angiogenesis (Dejana, 2004, Gentil-dit-Maurin et al., 2010). VE-Cadherin knockout mice died at 9.5 days of gestation due to vascular disintegration caused by increased endothelial apoptosis (Carmeliet et al., 1999). Similarly, injection of antibody (clone BV13) against VE-Cadherin into wild type mouse induced death after 24 hours due to vascular disassembly (Corada et al., 1999). All these studies are highlighting the importance of VE-Cadherin. Like other cadherins, VE-Cadherin also binds to a set of catenins at their cytoplasmic tails. The JMD of VE-Cadherin plays a key role in binding to P120-catenin (Ferber et al., 2002). An octapeptide (YDEEGGGE) in the JMD-VE-Cadherin is necessary for stable VE-Cadherin function and cell proliferation (Ferber et al., 2002). Moreover, P120-catenin binding to VE-Cadherin inhibits the clathrin-dependent internalization of VE-Cadherin (Xiao et al., 2005). VE-Cadherin found exclusively in vascular endothelial cells. Association of VE-Cadherin with β-catenin regulates cadherin phosphorylation and

down regulation upon exposure to inflammatory mediators such as thrombin (Vestweber, 2008). In addition, the VE-Cadherin/β-catenin complex forms an interaction with vascular endothelial growth factor receptor 2 (VEGFR2), which regulates VEGF induced endothelial cell function (Giannotta et al., 2013). Endothelial cells also express N-Cadherin. However' VE-Cadherin strongly localizes to adherence junctions, while N-Cadherin is expressed on all endothelial cell surfaces (Gentil-dit-Maurin et al., 2010, Liaw et al., 1990). The function of N-Cadherin on endothelial cells remains unknown. Giampietro *et al.* reported that VE-Cadherin negatively regulates the expression of N-Cadherin by decreasing the availability of P120-catenein (Giampietro et al., 2012). N- and VE-Cadherin show overlapping and divergent signaling pathways in endothelial cells (Giampietro et al., 2012).

Endothelial cells are the gatekeepers of vasculature, controlling the passage of plasma components and circulating cells from blood to the underlying tissues. The integrity of the endothelial cell barrier must be tightly regulated to prevent exposure of the subendothelial matrix of blood vessels to flowing blood, thereby preventing thrombotic events (Dejana, 2004, Vestweber, 2007). Due to the key role of VE-Cadherin at adherent junctions, several studies have focused exclusively on VE-Cadherin. Vascular permeability can be induced by sequential phosphorylation, cleavage and internalization of VE-Cadherin (Dejana et al., 2008) allowing the conclusion that VE-Cadherin is critical for the gatekeeper function of endothelial cells. Moreover, VE-Cadherin has been identified

as a target for inhibition of pathological angiogenesis using monoclonal antibodies (clone BV13 and BV14) (Corada et al., 2002). The potential role of anti-VE-Cadherin therapy was further shown using a cyclic peptide derived from the cell-adhesion recognition sequence present on the VE-Cadherin extracellular domain (Navaratna et al., 2008). These findings suggest agents that can target VE-Cadherin function in angiogenesis might be useful therapeutic agents to treat cancer or disorders of angiogenesis.

1.6 Angiogenesis

Angiogenesis is a physiological process, which forms new blood vessels from pre-existing vessels. This is essential for growth and tissue repair. Blood vessels allow the passage of blood cells around the body facilitating immune surveillance, supply of oxygen and nutrients and disposal of waste. Vessels induce instructive signals for organogenesis, which is beneficial for tissue growth and regeneration (Carmeliet, 2003). Deviation from normal vessel growth contributes to numerous diseases. In cancer, tumour cells produce angiogenic signals that help the tumour cells to metastasize by providing new blood vessels to supply nutrients for growing tumours. It is a systematic facilitation of angiogenesis that allows the spread of cancer that can eventually lead to patient death (Folkman, 2007). Insufficient vessel growth or repair of blood vessels also leads to myocardial infarction, stroke, neurodegeneration, pulmonary hypertension and blinding eye diseases (Carmeliet, 2003). Angiogenesis is a complex process composed of several signaling cascades that

support vessel growth and repair. Vascular endothelial growth factor (VEGF) is a major growth factor for stimulation of angiogenesis by signaling through Vascular endothelial growth factor receptor 2 (VEGFR2) (Ferrara, 2009). During angiogenesis endothelial cell migration is mainly regulated by three different mechanisms: chemotaxis, heptotaxis and mechanotaxis (Lamalice et al., 2007). Chemotaxis is movement of cell mediated by growth factors for example VEGF (Lamalice et al., 2007). Heptotaxis, the directional migration toward immobilized extracellular matrix proteins such as collagen (Lamalice et al., 2007). Endothelial cells line the interior of blood vessels and they are always in contact with fluid shear stress induced by blood supply. This shear force also helps the endothelial cell migration during angiogenesis (Lamalice et al., 2007), this process called mechanotaxis. VE-Cadherin is a key player in angiogenesis (Wallez et al., 2006). The major role of VE-Cadherin in angiogenesis is further explained in chapter 5.

1.7 Palmitoylated peptides

Palmitoylated peptide technology was initially used by Stephens *et al.* to modulate the function of the platelet cell adhesion molecule integrin αIIb (Stephens et al., 1998). Stephens *et al.* demonstrated the value of adding a lipid moiety to a bioactive peptide in order to render the peptide cell permeable (Stephens et al., 1998). The expectation was that conjugated peptides remained attached to the lipid moiety and thus made such peptides ideal for assessing function in the membrane-adjacent regions of transmembrane proteins. In addition, Covic *et al.* also used

palmitoylation technology to specifically target the PAR1 receptor activity in platelets and determined the cell-penetrating abilities of palmitoylated peptides using fluorescent-tagged palmitoylated peptides (Covic et al., 2002a). Moreover, Koloka *et al.* have demonstrated the platelet permeability of integrin αIIb-derived palmitoylated peptides using carboxyfluorescein-labeled palmitoylated peptide (Koloka et al., 2008). Together, these findings indicate that the palmitoylated peptides can localize to the intracellular membrane surface to exert their effects. Edwards *et al.* explored the ability of palmitoylated peptides, whose sequence was derived from evolutionary-conserved membrane adjacent regions of platelet transmembrane proteins, to modulate platelet activity (Edwards et al., 2007). Twenty-five peptides, from a panel of 83 transmembrane proteins were shown to exhibit bioactivity that could be predicted from knowledge of the function of the parent protein (Edwards et al., 2007). In addition, Bernard *et al.* showed that simple amino acid substitution within these biomimetic peptides could modulate their bioactivity (Bernard et al., 2009). Several subsequent studies suggested that conjugation of palmitate to peptides derived from key regions of signaling proteins allows modulation of downstream signaling activity (Covic et al., 2002b, Shpakov et al., 2007, Koloka et al., 2008). Using palmitoylation technology, various studies have developed platelet activatory peptides for specific receptors including platelet integrin αIIb (Stephens et al., 1998), PAR1 (Covic et al., 2002a), PAR2 (Sevigny et al., 2011), melanocortin-4 (MC4) (Covic et al., 2002a), chemokine CXC-type receptor 4 (CXCR4) (Tchernychev et al., 2010), sphingosine 1-phosphate

(S1P3) (Licht et al., 2003) and formyl peptide receptor-2 (FPR2) (Lee et al., 2010). In addition, palmitoylation technology was also used to develop platelet antagonist peptides from the intracellular region of various receptors including PAR1 (Covic et al., 2002b), PAR4 (Hollenberg et al., 2004), CD226 (Edwards et al., 2007), integrin αIIb (Koloka et al., 2008, Gkourogianni et al., 2013) and anti-angiogenic peptide from CXCR1/2 (Yang et al., 2009). Moreover, as evidenced by previous study from Edwards *et al.* libraries of palmitoylated peptides can be readily designed and tested for agonist and antagonist activity by high-throughput screening (Edwards et al., 2007). Together, these findings suggest that palmitoylated peptides can be used to explore the role of various receptors in platelet function and angiogenesis.

1.8 Aims

Identification and understanding of the function of new platelet proteins will have a potential impact on the development of novel therapeutic agents for the treatment of platelet disorders. There is evidence to suggest that a peptide derived from K-Cadherin is able to modulate platelet function (Edwards et al., 2007). In addition to this, K-Cadherin has been identified in platelets (Dunne et al., 2012). However, there is a lack of evidence to show the expression of other cadherins and their role in human platelets. The main aim of this thesis, therefore, is to explore the role of cadherins in human platelets and to examine the anti-platelet effects of peptides derived from various classical cadherins. As the project progressed I sought to gain a greater understanding of the role of

cadherins in human platelets. Additionally I investigated the effect of cadherin-derived palmitoylated peptides on endothelial cell function and assessed their ability to modulate the process of angiogenesis.

Chapter 2

Materials and Methods

2.1 Materials

All general reagents were purchased from Sigma Aldrich, Ireland and all other reagnets were listed in (Table 2.1). All palmitoylated peptides used in this thesis were custom synthesized from Peptide 2.0 USA with >85% purity.

Table 2.1 List and source of reagents used in this study

Material	Source
Angiogenesis kit	Millipore, Cork, Ireland
[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS Reagent)	Promega, UK
Accutase	Sigma Aldrich, Ireland
Anti-mouse FITC secondary antibody (488)	Bio-Sciences, Dun Laoghaire Co Dublin Ireland
Anti-mouse HRP linked secondary antibody	Millipore, Cork, Ireland
Anti-Rabbit HRP linked secondary antibody	Cell Signaling, supplied by Brennan and Company, Dublin, Ireland
ATP detection reaget (Chronolume)	Labmedics, Oxfordshire, UK
Bio-Rad protein assay kit	Life Science, Dublin, Ireland
Calcein AM	Sigma Aldrich, Ireland
Collagen	Brennan & Company, Dublin, Ireland
Dulbecco's Modified Eagle Media (DMEM)	Sigma Aldrich, Ireland
Falcon round bottom tubes	Becton-Dickinso, Oxford, UK
FCRγ antibody	Miltenyi Biotec Ltd, Surrey, UK
Fetal bovine calf serum (FBS)	Sigma Aldrich, Ireland
Flat bottom aggregation tubes (7.25X55cm)	Brennan & Company, Dublin, Ireland
Fluorescence Mounting medium	Dako Diagnostics Ireland Ltd
Geneticin di sulfate salt	Sigma Aldrich, Ireland
Gentamicin	Sigma Aldrich, Ireland
Geocin	Sigma Aldrich, Ireland
HUVEC complete media kit	Millipore, Cork, Ireland
Integrin αIIbβ3 (SZ22),	Santa Cruz biotechnology, Heidelberg, Germany
Lactate dehydrogenase (LDH) assay kit	Sigma Aldrich, Ireland
Non-specific mouse IgG	Santa Cruz biotechnology, Heidelberg, Germany

Material	Source
p-Nitrophenyl phosphate (pNPP)	Thermo Fisher Scientific, Ireland
Phalloidin TRITC	Sigma Aldrich, Ireland
Phosphotase inhibitor cocktail (100X)	Thermo Fisher Scientific, Ireland
Phosphotyrosine antibody (4G10)	Millipore, Cork, Ireland
Poly-L-lysine	VWR International Ltd, Dublin, Ireland
Polyvinyl difluoride (PVDF) membrane	Sigma Aldrich, Ireland
Protease inhibitor cocktail (100X)	Thermo Fisher Scientific, Ireland
Protein A agarose beads	GE Healthcare, Citywest Business Campus, Dublin, Ireland
Pure recombinant K-Cadherin	R&D systems, Abingdon, UK
Pure recombinant VE-Cadherin	R&D systems, Abingdon, UK
Supersignal West Pico chemiluminescent	Thermo Fisher Scientific, Ireland
Thrombin receptor activating peptide (TRAP)	R&D systems, Abingdon, UK
Trypsin	Promega, UK
U46619 (thromboxane mimetic)	R&D systems, Abingdon, UK
VE-Cadherin blocking antibody (BV9)	Millipore, Cork, Ireland
White 96-well plates	NUNC Thermo Fisher Scientific, Ireland

2.2 Methods

2.2.1 Preparation of washed platelets (WP)

Venous blood was drawn from healthy volunteers, who had not taken aspirin, or other drugs known to affect platelet function, for 7-10 days prior to phlebotomy. Ethical approval for blood collection was granted by the RCSI ethics committee and informed written consent was obtained from all volunteers. Blood was drawn using a 19 gauge butterfly needle by a qualified phlebotomist. 30 to 60ml of blood was drawn into syringe containing 15% of anticoagulant acid citrate dextrose (ACD) (38mM citric acid, 75mM sodium citrate, 124mM dextrose). Blood was aliquoted in 15ml tubes (5ml per tube) and centrifuged at 150Xg for 10 minutes at room temperature. This centrifugation step results in whole blood being

separated into two fractions. Platelet rich plasma (PRP) layers on top of a packed red cell pellet. The PRP was carefully transferred to a 50ml tube with a transfer pipette and the pH was adjusted to 6.5 with ACD. Prostaglandin E_1 (PGE_1), at a final concentration of 1μM, was added to prevent platelet activation during high speed centrifugation. The PRP was centrifuged at 720Xg for 10 minutes and the resulting platelet pellet was resuspended in buffer A (130mM sodium chloride, 10mM sodium citrate, 9mM sodium bicarbonate, 6mM dextrose, 0.9mM magnesium chloride, 0.81 potassium hydrogen phosphate 10mM Tris, and pH 7.4). The platelet count was adjusted to $3X10^5$/μl and allowed to rest for 45 minutes. WPs were supplemented with 1.8mM calcium chloride 10 minutes prior to use.

2.2.2 Platelet aggregation

Washed platelets (WPs) were prepared as described in section (2.2.1) and platelet aggregations were performed at 37°C using a Bio-Data PAP-8 aggregometer (platelet aggregation profiler, Horsham, PA, USA). 200μl of WPs were aliquoted into siliconised glass tubes. The compounds of interest (peptides, antagonists and agonists) were added to the platelets at various concentrations to give a final volume of 250μl. Platelets were incubated for 5 minutes with buffer, peptides, or antagonists. Aggregation was monitored for 5 to 10 minutes after the addition of agonists. During the aggregation, platelets were stirred at 1100 revolutions per minute (rpm) to promote platelet-platelet contact and subsequent platelet aggregate formation. Percentage of platelet aggregation was determined

by the percentage of light transmission through a sample of platelets in solution. As platelet aggregates are formed, they fall to the bottom of the tube thereby increasing the level of light transmission through the sample. A 100% aggregation baseline is set in the aggregometer by measuring the level of light transmission through buffer A.

2.2.3 Platelet adenosine triphosphate (ATP) secretion

Secreted ADP/ATP was measured using the method described by Sun *et al.* (Sun et al., 2001). Different concentrations of peptides or buffer in a final volume of 10µl were dispensed into white 96-well plates. To this, 70µl of WPs were gently added. Peptides and platelets were mixed by shaking (high speed, orbital shaking) of the plate at 37°C for 12 minutes in the Perkin Elmer 1420 96-well plate reader. Agonist was then added to induce platelet ADP/ATP release. The suspension (peptide + platelets + agonist) was then incubated for 3 to 6 minutes at 37°C with constant shaking. Finally, 10µl of ATP detecting reagent, chronolume was dispensed into the wells of the plate and luminescence was measured using the Perkin Elmer 1420 96-well plate reader (Ballymount, Dublin, Ireland). Platelet ADP/ATP secretion response to peptides was observed in the presence and absence of agonist. Data was expressed as the amount of ATP secretion in luminescence arbitrary units (AU). Data is represented as the mean ± standard error mean (SEM) of independent donors.

2.2.4 Red blood cell (RBC) lysis assay

The ability of toxins or peptides to cause lysis of suspensions of RBCs was used as an indication of toxicity (Saar et al., 2005). To prepare suspensions of RBCs, packed left-over RBCs were taken after the first spin of whole blood from the washed platelet preparation as described in section 2.2.1. 1ml of packed RBCs were tranferred to a 15ml tube and diluted with 9ml of buffer A. Diluted RBCs were centrifuged at 1000Xg for 5 minutes, supernatant was discarded and the RBC pellet was resuspended in fresh 10ml buffer A. The final volume of the assay was 300µl. Samples were dispensed in the following order: Buffer A, 50µM peptide or 0.1% TritonX100, and finally, 10%v/v of diluted RBCs. Samples were incubated at 37°C for 15 minutes. After incubation, samples were centifuged at 1000Xg for 5 minutes and the supernatant was analyzed for the presence of hemoglobin at absorbance 405nm using a Perkin Elmer 1420 96-well plate reader (Ballymount, Dublin, Ireland).

2.2.5 Lactate dehydrogenase (LDH) assay

The LDH assay kit was purchased from Sigma Aldrich, Ireland and assay was performed according to manufactures protocol. The LDH assay kit contains three different types of reagents such as substrate solution, cofactor solution and dye solution. For toxicity analysis these reagents should be mixed in equal proportions. The assay is based on the reduction of Nicotinamide adenine dinucleotide (NAD) by LDH. The resulting reduced NAD (NADH) is utilized in the stoichiometric conversion

of a tetrazolium dye. The resulting colored compound is measured spectrophotometrically. WPs were treated with various peptides or lysis buffer or platelet buffer for 15 minutes at 37°C. After incubation, samples were centrifuged for 3 minutes at 720Xg and 50µl of supernatant was carefully transferred to clear flat bottom 96-well plates. To each well containing supernatant, 100µl of mixture of the 3 LDH reagents were added and the plates were incubated in the dark for 20 minutes at room temperature for colour development. Following incubation of the samples in the dark, absorbance values were measured at 490nm using a Perkin Elmer 1420 96-well plate reader. All the samples were measured in duplicates, along with a positive control (0.1% Triton) and a negative control (buffer).

2.2.6 Western blotting

Western blotting is a technique widely used to identify proteins in a sample or to identify post translational modification of a protein, such as phosphorylation (Towbin et al., 1979). Initially a mixture of proteins is separated according to their molecular weight by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins are transferred from gel to polyvinyl difluoride (PVDF) membrane. The PVDF membrane is blocked to ensure a uniform protein surface and prevent the non-specific binding of antibodies and subsequently probed with specific antibody to protein of interest. Then the membrane is washed and reprobed with secondary antibody which is conjugated to a reporter enzyme, such as horseradish peroxidase (HRP),

which enhances the signal during the blot development through chemiluminescence detection technique.

2.2.6.1 Preparation of platelet lysates

Washed platelets were prepared as described in section 2.2.1 and the count was adjusted to $1X10^6$/µl. The high platelet count was used to extract sufficient concentration of protein for western blot analysis. Platelets were unactivated and activated with either TRAP or collagen or U46619. The samples for electrophoresis were prepared using the platelet aggergation assay. Platelet aggeragation was performed as described in section 2.2.2. At various time points in the aggregation, 10X platelet lysis buffer (500µM N-Octyl glucoside, 25mM phenylmethylsulfonyl fluoride (PMSF), 10% Trition, 1mM Ethylenediaminetetraacetic acid (EDTA), 1% Sodium dodecyl sulfate (SDS), 50mM Tris (pH7.8) and a 1 in 10 dilution of 100X protease and phosphatase inhibitors) was added to the aggregation tubes. Lysis buffer was at a final concentration of 1X in the platelet lysate samples. The mixture of platelets and lysis buffer was incubated at 4°C under agitation for 1 hour. The platelet lysates were subsequently centrifuged at 10,000Xg for 10 minutes. The resulting supernatants were transferred to new eppendorfs and these lysates were used immediately or divided into aliquots and stored at -80°C.

2.2.6.2 Determination of protein concentration

The protein concentration of platelet lysates was determined by the Bradford method (Bradford, 1976) using with Bio-Rad protein assay kit. In brief, 5µl of platelet lysate was mixed with 25µl of reagent A (BioRad protein assay) in a clear 96-well plate. 200 µl of reagent B was added to each well. Following addition of reagent B, samples were incubated at room temperature for 15 minutes and the absorbance of each well was subsequently measured at 595nm using Perkin Elmer 1420 96-well plate reader. The protein concentration was calculated using a standard curve generated with bovine serum albumin (BSA) concentrations ranging from 0.2 to 1.5mg/ml.

2.2.6.3 Immunoprecipitation analysis of platelet proteins

VE- and K-Cadherins were seperated from platelets by immunoprecipitation. Initially, protein A agarose beads were blocked with 1% BSA for 1 hour at 4°C. Platelet lysates were prepared as described in section 2.2.6.1 and pre-cleared with protein A beads before incubation with antibody. Protein A beads, 4µg/ml antibody (VE, K- Cadherin and non-specific mouse IgG antibodies) and pre-cleared platelet lysates were incubated together overnight at 4°C. After incubation, beads were washed 5 times with TBS-T (20mM Tris-base, 300mM sodium chloride and 0.1% tween). Proteins attached to the beads were separated using 2X sample buffer (4% SDS, 10% Dithiothreitol (DTT), 20% glycerol, 0.125M Tris and 0.1% bromophenol blue) and samples were analyzed by SDS-PAGE.

2.2.6.4 SDS polyacrylamide gel electrophoresis

SDS-PAGE is a method to separate the complex of proteins according to molecular weight under denaturing conditions as described by laemmli *et al.* (Laemmli, 1970). SDS-PAGE mini gels, 7.5% were prepared in sealed 1.0mm Bio-rad glass plates. The final volume of 14ml of 7.5% resolving gel solution was prepared by mixing of 3.5ml resolving buffer (1.5M Tris, pH7.8, 0.4% SDS), 3.5ml of 30% acrylamide and 7ml of dH_2O, 82µl 10% (w/v) ammonium persulphate (APS) and 7.8µl of tetramethylenediamine (TEMED). The resolving gel poured to approximately 2.5cm from the top of the glass plate. Approximately 1ml of methanol was placed on top of the gel to avoid air bubbles. The gel was allowed to polymerise for 1 hour at room temperature and methanol was washed using dH_2O after solidification of the gel. The final volume 16ml of 4.5% stacking gel solution was prepared by mixing of 3.75ml of stacking buffer (0.5M Tris, pH6.8, 0.4% SDS), 2.25ml of 30% acrylamide and 9ml of dH_2O. Immediately before pouring the stacking gel 110µl of 10% (w/v) APS and 15µl of TEMED were added to the stacking gel solution. After washing of methanol from resolving gel, the stacking gel was gently poured on top of the resolving gel and a 10 lane (1.0mm) comb was carefully inserted, at an angle in order to avoid forming air bubbles. The gel was left to polymerise for 1 hour at room temperature.

After preparation of SDS-gels, platelet lysates were prepared to run in SDS-gels. The protein concentration of platelet lysates was determined using the Bio-Rad protein assay kit as described in section 2.2.6.2.

Following protein concentration determination, the volume required for equal loading of 20µg protein per lane was calculated. This volume of platelet lysate was incubated with 5X reducing sample buffer (312.5mM Tris, 5%w/v SDS, 20% w/v glycerol, 2%w/v DTT, 0.25% w/v bromophenol blue) such that the final concentration of sample buffer was 1X and samples were boiled at 95°C for 5 minutes. Samples were loaded onto SDS-PAGE in respective lanes along with 10µl of molecular weight standards in seperate wells as a reference to indicate the relative size of the proteins. The gels were setup for the electrophoresis tank with running buffer (25mM Tris-Base, 190mM glycine and 0.1% SDS) and run at a constant voltage of 100V for approximately for 1 hour 30 minutes.

2.2.6.5 Visualization of proteins on SDS-PAGE

To visualize the proteins on SDS-PAGE, gels were stained with Coomassie Brilliant Blue-250 (CBB-250) stain (Dyballa and Metzger, 2009). Following protein seperation by SDS-PADE, resolving gels were removed from glass cassettes and the stacking gels were discarderd. The resolving gels were stained with Coomassie solution [0.02% CBB-250, 5% aluminium sulfate, 10% ethanol, 2% Orthophosphoric acid (85%)] for 24 hours at room temperature and then destained using destaining solution [10% ethanol, 2% Orthophosphoric acid (85%)] for 24 hours at room temperature. After destaining, gels were stored in distilled water at 4°C. Images of the gels were captured on a UVP bio imaging system (Davidson & Hardy Laboratory Supplies Ltd, Dublin, Ireland) and stored as pdf files.

2.2.6.6 Protein transfer and development of Western blots (Towbin et al., 1979)

Following protein seperation by SDS-PAGE, gels were removed from glass cassettes and stacking gels were discarded. The proteins present in the resolving gels were transferred to a PVDF membrane using the Bio-Rad Wet blot transfer cell apparatus with 1X Transfer buffer (25mM Tris-Base, 190mM glycine 0.04% SDS and 20% methanol). Resolving gels were sandwiched between PVDF, 4 sheets of 3MM filter paper (Whatman, Sigma Ireland) and two sponges in the following order from the bottom of the electrode plate. Sponge, two pieces of filter paper, resolving gel, one piece of PVDF membrane, two pieces of filter paper and another sponge. PVDF membranes were activated with methanol and sponges, were presoaked in transfer buffer prior to preparation of sandwich. The holder containg resolving gel and PVDF membrane was placed into the transfer apparatus and the transfer was performed in the presence of ice block at constant 100V for 1 hour using a Bio-Rad power pack. After transfer, the PVDF membranes were blocked for 1 hour with 3% BSA dissolved in TBS-T (20mM Tris-Base, 137mM Sodium chloride, 0.1% Tween and pH 7.4). Blocked membranes were incubated with primary antibodies of interest for overnight at 4°C (the concentrations of antibodies used were listed in Table 2.2). Following overnight primary antibody incubation, unbound primary antibodies were removed by washing the membranes in TBS-T (3 X 5 minutes at room teperature). Following this, membranes were incubated with appropriate secondary antibodies for 1 hour at room temperature. After incubaton, non-

specifically bound secondary antibody was removed by washing the mebrane with TBS-T (3 X 10 minutes at room temperature). Membranes were treated with enhanced chemiluminescence ECL solution and bands or signals were measured using a western blot developer, Bio imaging system UVP (Davidson & Hardy Laboratory Supplies Ltd, Dublin, Ireland).

Table 2.2 List of antibodies, source and concentrations used for western blots.

Primary antibody (source)	Dilution	Molecular Weight (kDa)	Secondary antibody
E-Cadherin (Cell Signaling, Brennan and Company, Dublin, Ireland)	1 in 1000	135	Anti-rabbit
N-Cadherin (Cell Signaling)	1 in 1000	140	Anti-rabbit
VE-Cadherin (e-bioscience Hatfield, UK)	1 in 1000	140	Anti-mouse
K-Cadherin (R&D systems, Abingdon, UK)	1 in 1000	120	Anti-mouse
Pan-Cadherin (Cell Signaling)	1 in 1000	120	Anti-rabbit
P120-catenin(Cell Signaling)	1 in 1000	100	Anti-rabbit
β-catenin (Cell Signaling)	1 in 1000	92	Anti-rabbit
Junctional plakoglobin (Millipore, Cork, Ireland)	1 in 1000	80	Anti-mouse
α-catenin (Cell Signaling)	1 in 1000	100	Anti-rabbit

2.2.7 Preparation of gels for Mass spectrometric (MS) analysis

Following immunoprecipitation of platelet proteins, samples were resolved using electrophoresis as described in section 2.2.6.4 and stained with Coomassie blue as described in 2.2.6.5.

2.2.7.1 Excising bands from the gel

Individual bands on the resolved gel were cut out using a scalpel. The excised bands were chopped into small pieces and the gel pieces were

placed in small eppendorfs containing 70µl of 200mM ammonium bicarbonate (NH_4HCO_3, pH 7.8).

2.2.7.2 Shrinkage of the bands

Gel pieces were shrank by placing the eppendorfs in a thermomixer at 37°C, 1000 rpm, for 15 minutes. The samples were then centrifuged at 10,000 rpm for 10 seconds and the resulting supernatant was discarded. The samples were treated with three different reagents. Firstly, 70µl 200mM NH_4HCO_3/acetonitrile (4:6) mixture was added to each sample, and centrifuged at 10,000 rpm for 10 seconds and the supernatant was dicarded. Secondly, the samples were incubated with 70µl of 50mM NH_4HCO_3 (pH 7.8) for 30 minutes at 37°C, centrifuged at 10,000 rpm for 10 seconds and the supernatant was discarded. Finally, samples were treated with 70µl of MeCN for 5 minutes at room temperature, centrifuged at 10,000 rpm for 10 seconds and the supernatant was discarded.

2.2.7.3 Reduction, alkylation, and dehydration of Mass spectrometry samples

After shrinkage, samples were reduced and alkylated. 50µl of 10mM DTT (dissolved in 100mM NH_4HCO_3) was added to each sample and incubated for 1 hour at 56°C. After incubation, DTT was removed by centrifuging the samples at 10,000 rpm for 10 seconds. The samples were alkylated by incubating them in the dark for 30 minutes with 50µl of 50mM Iodoacetamide (dissolved in 100mM NH_4HCO_3). After alkylation samples were washed twice with 200µl of 100mM NH_4HCO_3. NH_4HCO_3

was removed by centrifuging the samples at 10,000 rpm for 10 seconds. Then the samples were dehydrated by treating them with 70µl of Acetonitrile (MeCN) for 5 minutes at 37°C and dried using Speedvac (Eppendorf UK Limited).

2.2.7.4 Trypsin digestion

Following dehydration, samples were digested with trypsin. Trypsin solution was prepared by dissolving 2µg of trypsin in 250µl of 50mM NH_4HCO_3/1mM calcium chloride ($CaCl_2$). The final concentration of 720ng trypsin was added to the each sample in the following order. Initally 240ng of trypsin was added to each dry sample, waited for 5 minutes and another 480ng of trypsin was added. Then the samples were incubated at 37°C at 1000rpm in a themomixer (Eppendorf UK Limited) for overnight.

2.2.7.5 Peptide extraction

After overnight incubation of samples with trypsin, digested peptides were extracted in three steps. Firstly, 10µl of 5% Trifluoroacetic acid (CF_3CO_2H) was addded to each sample, vortexed thoroughly and incubated for 2 minutes at room temperature. Samples were centrifuged at 10,000 rpm for 10 seconds and supernatant was transferred into individually labelled 500µl eppendorfs. Secondly, 20µl mixture of 2% CF_3CO_2H/60%MeCN was added to each sample, vortexed thoroughly and samples were incubated for 10 minutes at room temperature. The hydrophobic peptides were eluted by centrifuging the samples at 10,000

rpm for 10 seconds and supernatants were transferred into individually labelled eppendorfs. Finally, the remaining peptides were eluted with MeCN. 20µl of 100% MeCN was added to each sample, vortexed thoroughly and incubated for 5 minutes at room temperature. The samples were centrifuged at 10,000 rpm for 10 seconds and supernatant was transferred to individually labelled eppendorfs. Before analyzing the samples in MS, three different elutes of each sample was combined into a single eppendorf tube. Then the samples were vacuum centrifuged until a droplet remained and 20µl of 0.1% Trifluroacetic acid (TFA) was added before being transferred to high performance liquid chromatography (HPLC) vials for Orbitrap analysis. 4µl of this was analysed by Liquid chromatography–mass spectrometry (LCMS) (Thermofisher, UK).

2.2.7.6 HPLC conditions

Peptides were concentrated on a Pepmap C-18 trap column (300µm ID x 5mm) and separated on a Pepmap C18 reversed phase column (Dionex, UK) (3µm particles, 75µm ID x 250mm) using a linear gradient for 97 minutes from 96% A (0.05% formic acid), 4% B (0.05% formic acid, 80% acetonitrile) to 60% A, 40% B, and for 5 minutes from 60% A, 40% B to 35% A, 65% at a flow rate of 300 nl/min.

2.2.7.7 Orbitrap Mass spectrometry conditions

Using Xcalibur 2.0.1 (Thermo scientific, UK), intact peptides were detected between m/z 400 and m/z 1,600 in the Orbitrap XL (Thermo scientific, UK) at a resolution of 30,000. Internal calibration was

performed using the ion signal of (Si(CH3)3O)6H+ at m/z 445.120025 as a lock mass (Olsen et al., 2005). Parts per million mass accuracy on an Orbitrap mass spectrometer (Thermo scientific, UK) via lock mass injection into a C-trap. Maximum ion accumulation time allowed on the LTQ Orbitrap was 50ms for FT MS and 1s for ion trap MSMS. Automatic gain control was used to prevent over-filling of the ion trap. Collision induced dissociation (CID) spectra of the top 5-peptide ions (rejection of singly charged precursors) were acquired between m/z 400 and m/z 1,600 at normalised collision energy of 35. Dynamic exclusion was set with a repeat count of 1, a repeat time of 30 seconds, and an exclusion time of 3 minutes. The chromatography feature was enabled with a correlation area ratio of 1.0. Activation Q was set to 0.25 with 30 minutes activation time. A list of m/z values for VE- and K-Cadherin peptides was incorporated into the method to preferentially trigger MSMS on those peptides. Cytochrome C digests were run as a standard sample before and after the digests to assess LCMS performance.

2.2.7.8 Data analysis

Raw data was transformed into peak lists in the mascot generic file format (MGF) using Proteome Explorer, version 1.1 (Applied Biosystems, USA) and default settings. The MGF file was submitted to a local installation of the X! Tandem search engine using the Global Proteome Machine (GPM) interface (www.thegpm.org) and the following parameters. Tryptic digestion with up to one missed cleavage site, precursor ion tolerance ±20 ppm, product ion tolerance 0.4 Da,

carboxamidomethylation of Cys as a fixed modification and Oxidation of Met as a variable modification. The following additional variable modifications were considered in two refinement stages. Phosphorylation of Ser, Thr and Tyr, oxidation of Met and Trp, methylation of Cys, Asp, Glu, His, Lys and Arg and deamidation of Asn and Gln in the first refinement stage and di-oxidation of Met and Trp, dehydration of Ser and Thr, lack of carboxamidomethylation of Cys, methylation of Asn and Gln, carboxamidomethylation of Lys, His, Glu and Asp in the second refinement stage. The ENSEMBL GRCh37.55, taxonomy human and the common repository for adventitious proteins (cRAP) version 2009.05.01 were searched.

2.2.8 Densitometry analysis

Western blot band intensities were analysed using Image J software (http://rsbweb.nih.gov/ij/). A small rectangle was drawn to measure the intensity of each Western blot band resulting in a peak. The area under the peak was then measured. The ratio of protein expression was calculated by area of protein of interest divided by area under the peak of the loading control band. For VE-Cadherin and K-Cadherin expression, area of each band was normalised to the area for positive control band as 100%.

2.2.9 Bioinformatic analysis

For the identification of conserved residues among the cadherins, a multiple protein sequence alignment was generated using Muscle (Edgar, 2004). SLIMs were identified using SLIM prediction analysis (Mooney et al., 2012). Control peptides to parent peptides were designed using (http.//bioware.ucd.ie/~cyclops/Fergal/tags/PepControls).

2.2.10 Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were cultured in EndoGRO-LS medium (Millipore, Cork, Ireland) containing 0.2% EndoGRO-LS Supplement, 5ng/ml Endothelial growth factor (EGF), 50µg/ml ascorbic acid, 10mM L-Glutamate, 1µg/ml hydrocortisone hemisuccinate, 0.75U/ml heparin sulfate, and 2% fetal bovine calf serum (FBS). Cells were grown in T175 cm^2 flasks at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. Cells were allowed to grow to 70 to 80% confluence and then subsequently sub-cultured. Cell passage numbers from 2 to 5 were used for all experiments.

2.2.11 Angiogenesis assay

The capillary tubule formation assay was used to assess the effect of VE-Cadherin peptides on angiogenesis. For the assay, Matrigel solution (Millipore, Cork, Ireland) was thawed at 4°C and subsequently diluted in a 9:1 ratio (900µl of Matrigel and 100µl of diluent). To prevent the solidification of the Matrigel at room temperature during use, all pipette

tips and 96-well plates were pre-cooled to 4°C. Wells were coated with 50µl of Matrigel solution and the Matrigel was then allowed to solidify for 1 hour at 37°C. HUVECs were harvested and $2x10^4$ cells/well were seeded onto the Matrigel surface with complete HUVEC media containing 10ng/ml VEGF in the presence or absence of peptides. After 12 hours of incubation, images of each well were taken from 3 random fields. The number of capillary tubules formed was counted manually from each photograph and the length of tubule formation was analysed using ImageJ (http://rsbweb.nih.gov/ij/).

2.2.12 Wound healing assay

The wound-healing assay was also used to assess the effect of VE-Cadherin peptide on endothelial cells. HUVECs were grown in 6-well plates. Once cells are confluent, complete medium was replaced with serum free media and the incubation was continued for overnight. Following this, a linear scratch was made across the monolayer of cells with a sterile tip and detached cells were washed away with phosphate buffer saline (PBS) for 3 times. A set of 10x magnification images was taken using an inverted light microscope attached to a Zeiss camera (Zeiss) from three different random areas across the wound. HUVECs were subsequently incubated with complete HUVEC media containing 10ng/ml VEGF in the presence and absence of VE-Cadherin peptides. After 12 hours incubation at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide, a second set of images was taken from same

wound area. The number of cells that had migrated towards the wound area was calculated using ImageJ software.

2.2.13 Cell proliferation assay

The MTS [(3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) Promega, UK] cell proliferation assay was used to assess the effect of VE-Cadherin derived peptides on endothelial cell growth and proliferation. In this colorimetric assay, Nicotinamide adenine dinucleotide (NADH) in live cells converts tetrazolium salt to soluble formazan crystals. When confluent, HUVECs were detached, washed, and 5000 cells were added to 300µl final volume of HUVEC media with 10ng/ml VEGF. HUVECs were allowed to grow in clear 48-well plates in the presence or absence of peptides for 24 and 48 hours at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. After incubation, 20µl of MTS reagent was added to each well and the plates were returned to the incubator. After two hours of incubation with the MTS reagent, 100µl of supernatant was carefully transferred to a clear 96-well plate and the absorbance was measured at 490nm using Perkin-Elmer 1420 (Perkin Elmer, Ireland) 96-well plate reader.

2.2.14 Platelet adhesion to endothelial cells

To assess platelet adhesion to endothelial cells, HUVEC cells were grown in 2% gelatin coated 96-well plates. For inhibitory studies endothelial cells were incubated with various receptor antagonists for 30

minutes at 37°C prior to the assay. Washed platelets were prepared and labelled with 2µg/ml Calcein AM (Sigma, Ireland) for 15 minutes in the dark. Platelets were activated with 0.5U/ml thrombin (Sigma, Ireland) for 10 minutes and inactivated by adding 2U/ml hirudin for 10 minutes following activation. For inhibitory studies, platelets were also pre-treated with various inhibitors for 30 minutes at 37°C followed prior to platelet activation with thrombin. 100µl of pre-labelled resting or activated platelets were allowed to adhere to endothelial cells for 0 to 60 minutes at 37°C. Following this, unbound platelets were removed by washing three times with buffer A. Adherent platelets were lysed with 100µl of lysis buffer (150mM sodium chloride, 10mM Tris HCL P^H 7.4, 5mM EDTA, 10mM PMSF, 1% NP40 and 20µg/ml soybean trypsin inhibitor). After 10 minutes incubation with lysis buffer, the fluorescence signal of adherent platelets was measured at excitation 485 and emission 516 using a Perkin Elmer 1420 (Perkin Elmer, Ireland) 96-well plate reader.

2.2.15 Platelet adhesion assay

Clear flat bottom 96-well plates were coated overnight at 4°C with fibrinogen or BSA, or recombinant VE-Cadherin. After coating, wells were blocked with 1% BSA for 1 hour at 37°C. WPs were prepared as described in section 2.2.1. Platelets were pre-treated with different receptor antagonists before adding to the wells. 50µl of treated or non-treated platelets were allowed to adhere to immobilized proteins for 45 minutes at 37°C. After incubation, the wells were gently washed to remove non-adherent platelets. Adherent platelets were quantified using

an acid phosphatase assay. 100µl of 70mM sodium citrate, 30mM citric acid, 0.1% Triton and 5mM of para-nitrophenyl phosphate (PNPP) (Thermofisher scientific, Ireland) was added to each well and incubated for 45 minutes at 37°C. The reaction was terminated by the addition of 100µl 2M sodium hydroxide. The absorbance of each well was measured using a Perkin Elmer 1420 (Perkin Elmer, Ireland) 96-well plate reader at 405 nm.

2.2.16 Preparation of slides for confocal imaging

Clear microscope slides were coated with 3% BSA or 20µg/ml fibrinogen, or 1µg/ml recombinant VE-Cadherin for overnight at 4°C. After coating, slides were blocked with 1% BSA for 1 hour at 37°C to reduce non-specific binding. WPs were prepared as described in section 2.2.1 and diluted to $3X10^4$/µl using buffer A. 200 to 300µl of diluted washed platelets were added on to the immobilized protein surface and allowed to spread for 45 minutes at 37°C. Slides were washed three times with buffer containing 2mM calcium and platelets were fixed with 3% para formaldehyde (PFA) for 10 minutes at room temperature. Fixed platelets were washed 3 times with buffer A and permeabilized with 0.1% Triton in buffer A for 15 minutes at room temperature. Fixed and spread platelets were then stained with Phalloidin- Tetramethylrhodamine isothiocyanate (TRITC) (sigma, 1:100 dilution of stock) stain for actin. Platelets were incubated with phalloidin-TRITC for 30 minutes at room temperature. After staining, slides were washed 3 times with buffer A (130mM sodium chloride, 10mM sodium citrate, 9mM sodium bicarbonate, 6mM dextrose,

0.9mM magnesium chloride, 0.81 potassium hydrogen phosphate 10mM Tris, and pH 7.4), a cover slip was mounted on each slide using fluorescence mounting medium. Prepared slides were stored at 4°C and analysed within 5 days using a Zeiss LSM 510 Laser Scanning Confocal Microscope (Carl Zeiss Microscopy, UK).

2.2.17 Chinese hamster ovary (CHO) cell culture

CHO cells stably expressing platelet integrin αIIbβ3 as previously described by Aylward *et al.* (Aylward et al., 2006) were used in this study. Three cell lines were employed, a mock transfected CHO cell line, a CHO cell line expressing αIIbβ3 with KVGFFKR sequence in a resting conformation, and CHO cell line expressing αIIbβ3 with KVGAAKR sequence in an active conformation. All cell lines were grown in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine calf serum (FBS), 1% Gentamicin, 375μg/ml Geneticin di sulfate salt, and 250μg/ml Geocin. Cells were grown to 80-90% confluence and either used in experiments or split for further culturing. Accutase ® (3ml) solution was used for splitting as an alternative to trypsin in order to ensure that the integrin expressed in the CHO cells remained intact.

2.2.18 Assessment of platelet integrin αIIbβ3 levels in CHO cells

When confluent, CHO cells were detached with accutase and transferred to 15ml falcon tubes. Cells were centrifuged at 1000Xg for 3 minutes. Cell culture media was removed and pellet was resuspended in buffer A

(130mM sodium chloride, 10mM sodium trisodium citrate, 9mM sodium bicarbonate, 6mM dextrose, 0.9mM magnesium chloride, 0.81 potassium hydrogen phosphate 10mM Tris, and pH 7.4) to the desired concentration of $3.0X10^6$/ml. The levels of integrin expression in CHO cells were checked by flow cytometry using a monoclonal antibody against CD41a (αIIb subunit) conjugated to a FITC fluorophore. Cells were incubated with 10µl of anti-CD41a antibody for 15 minutes at room temperature. The reaction was stopped by adding 1ml of buffer A (130mM sodium chloride, 10mM sodium citrate, 9mM sodium bicarbonate, 6mM dextrose, 0.9mM magnesium chloride, 0.81 potassium hydrogen phosphate 10mM Tris, and pH 7.4) in 5ml Falcon tubes. Samples were analysed on Becton Dickinson FACScalibur flow cytometer (BD Biosciences, UK) using CellQuest Pro software (BD Biosciences, UK). The CHO cell population was gated and 5000 events were analysed.

2.2.19 CHO cell adhesion to immobilized protein

Clear 96-well flat bottom plates were coated with 100µl of 1µg/ml recombinant VE-Cadherin or 3% BSA overnight at 4°C. Excess substrate was removed and wells were blocked with 1% BSA for 1 hour at 37°C. After blocking, wells were washed once with buffer A supplemented with 1.8mM calcium chloride. 50µl ($2X10^6$/ml) of 2µg/ml calcein AM loaded CHO mock, CHO FF and CHO AA cells were allowed to adhere to immobilized proteins for 45 minutes at 37°C. Each condition was performed in duplicate. After incubation, non-adherent CHO cells were removed and washed twice with buffer A. Adherent cells were lysed with

100µl of lysis buffer (150mM sodium chloride, 10mM Tris HCL pH 7.4, 5mM EDTA, 10mM PMSF, 1% NP 40 and 20µg/ml soybean trypsin inhibitor). After 10 minutes incubation with lysis buffer, the fluorescence of adherent cells was measured at excitation 485 and emission 516 using a Perkin Elmer 1420 (Perkin Elmer, Ireland) 96-well plate reader. Data was expressed as arbitrary fluorescent units. For inhibitory studies, CHO cells were pre incubated with inhibitors for 15 minutes at 37°C before adding to the wells.

2.2.20 Identification of VE-Cadherin on platelets by flow cytometry

To assess the expression of VE-Cadherin on platelets, washed platelets ($3X10^5$/µl) were pre-treated with FCRγ antibody to avoid non-specific binding of antibodies (Buchwalow et al., 2011). After incubation with FCRγ antibody, platelets were incubated with 1:50 dilution of VE-Cadherin or 1:50 dilution of integrin αIIbβ3 (SZ22), or 1:50 dilution of non-specific IgG antibodies for 30 minutes. After incubation, platelets were treated with 1:200 dilution of secondary fluorescently labelled (Alexa Flour® 488) for 15 minutes. The reaction was terminated by the addition of 1ml of buffer A. Samples were analysed using FACS Canto (BD Biosciences, UK). Platelets were gated and at least 10,000 events were analysed.

2.2.21 Regression analysis of selective peptides

The activity of peptide association with different variables of peptide was calculated with regression analysis using STATA package

(www.stata.com). Different variables considered from each peptide are solubility, count of positive AAs at first and third position, number of positive and negative AAs in the whole peptide and percentage of hydrophobicity of the whole peptide. For regression analysis data was converted in the following manner. For the solubility, peptides that are soluble in water described as 1 and DMSO soluble peptides as 0. Active peptide represented as 1 and inactive peptide represented as 0. For the count of charged residues Arginine (R), Lysine (K) were considered as positively charged AAs and Aspartic acid (D) and Glutamic acid (E) were considered as negatively charged. Percentage of hydrophobicity was calculated based on number of hydrophobic AAs in each peptide.

2.2.22 Statistical analysis

All statistical analysis was performed using GraphPad Prism 5 (http://www.graphpad.com). Results are shown as mean ± standard error mean (SEM). Statistically significant differences between means were determined using one-way ANOVA, with post-hoc analysis using the Bonferroni test. P values *<0.05, ***<0.001 and ***<0.0001 were considered to be statistically significant.

Chapter 3

Design and analysis of Cadherin-derived Peptides in Platelet Function

3.1 Introduction

Inappropriate activation of platelets within an intact blood vessel may result in the formation of a clot leading to thrombotic events which exacerbate a number of cardiovascular diseases including angina, myocardial infarction, cardiac ischemia and thrombotic stroke. There is an ongoing search for pharmaceutical agents that can inhibit these thrombotic events. Synthetic peptides derived from functional motifs of human platelet proteins can both mimic and inhibit aspects of platelet function (Stephens et al., 1998, Covic et al., 2002b, Larkin et al., 2004, Kamb and Teng, 2000, Dimitriou et al., 2009, Mitsios et al., 2006, Edwards et al., 2007) and these peptides have been used in drug development (Scarborough et al., 1991). In 2007, Edwards and colleagues used a bioinformatic approach to identify peptides, which modulate platelet function (Edwards et al., 2007). The rationality behind Edwards *et al.* was Stephens *et al.* study, where they showed that an evolutionary highly conserved peptide sequence from signaling rich juxtamembrane domain (JMD) of integrin αIIb was able to induce the platelet activation (Stephens et al., 1998). This study suggests similar evolutionary-conserved peptides from JMD regions of other platelet proteins might be a rich source of bioactive peptides. Such peptides could yield great insights into platelet function. Similar to the Stephens *et al.* study, Edwards *et al.* designed peptides from evolutionarily highly conserved signaling rich JMD of transmembrane human platelet proteins and employed a systematic high-throughput assay to explore the potential of these peptides to act as platelet agonists and antagonists

(Edwards et al., 2007). Interestingly, a peptide derived from the JMD of the single-pass transmembrane protein K-Cadherin was identified in this initial screen as a potential platelet antagonist.

Therefore, the aim of the present study is to assess the ability of a range of cadherin-derived peptides to potentially interfere with human platelet function. Our approach, as performed in the Edwards *et al.* paper (Edwards et al., 2007), was to render peptides cell-permeable by attachment of a lipid moiety, such as palmitate, to the peptide sequence. This study highlights the previously unknown role of cadherins in platelet function.

3.2 Results

3.2.1 Design of peptides from cadherins

The cytoplasmic regions of classical cadherins are highly conserved and are known to play a role in maintaining the cadherin expression at the cell surface (Harris and Tepass, 2010). Cadherins bind to two different proteins, P120 and β-catenin, through their cytoplasmic tails. The JMD of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening and interaction with P120-catenin (Harris and Tepass, 2010). In the current study, peptides were designed from the JMD of E- and N-Cadherins using a similar approach to that of Edwards *et al.* (Edwards et al., 2007).

Disordered regions in protein structure are known to play a critical role in their function (Dyson and Wright, 2005). Short linear motifs (SLIMs) are functional sites present in disordered regions (Fuxreiter et al., 2007). SLIMs can be identified using various methods such as sequence alignment (Davey et al., 2012), to find common motifs in different proteins (Davey et al., 2011) and based on structural, biophysical, and biochemical features derived from the protein primary sequence (Mooney et al., 2012). NPXY is an example of a SLIM, which plays a key role in the binding of Kindlin-1 and 2 proteins to integrin β1 tails and regulates integrin β1 function (Harburger et al., 2009). Similarly the octapeptide YDEEGGGE is another example of a SLIM, located in VE-Cadherin juxtamembrane domain (JMD), which is crucial for the VE-Cadherin-P120-catenin interaction (Ferber et al., 2002). SLIMs play key roles in several biological processes including cell signaling, post-translational modifications and protein trafficking (Davey et al., 2010). Previously, Edwards *et al.* (Edwards et al., 2007) identified SLIMs from disordered JMD regions of platelet proteins to target the platelet function. In particular, one of the peptides derived from K-Cadherin, was identified as a platelet function modifier. In this chapter, our approach is further extended to generate a second generation of cadherin-derived JMD peptides to explore the role of cadherins in human platelet function.

SLIM analysis identified groups of residues in JMD of E- and N-Cadherins that are relatively conserved (Figure 3.1 and 3.2) (Mooney et al., 2012). Multiple sequence alignment of human cadherins (Figure 3.3b)

suggested that cadherins are highly conserved in their JMD, especially within the P120-catenin binding region. The interaction between the JMD of cadherins and P120-catenin is crucial for cadherin function (Anastasiadis and Reynolds, 2000). Overlapping peptides from the JMD of E-and N-Cadherins (Figure 3.3c) were designed and synthesized. In addition, peptides from K-Cadherin, as described in Edwards *et al.* were also synthesized. The peptides were N-terminally palmitoylated (pal) to facilitate tethering of peptide to the plasma membrane (Edwards et al., 2007, Covic et al., 2002b, Aylward et al., 2006, Koloka et al., 2008). A Ttds (1-amino-4,7,10-trioxa-13-tridecanaminesuccinimic acid) linker between the palmitic acid and the peptide sequence was used in E-Cad 3 and E-Cad 4 peptides to mimic the distance of the parent sequence from the plasma membrane. The peptides were named with letters and numbers. The letters refer to the parent cadherin (E, N or K) and the numbers refer to the position of the peptide relative to the plasma membrane. Thus, peptides close to the membrane are referred to as peptide 1 (e.g. E-Cad 1), with increasing peptide numbers corresponding to further distance from the membrane. Peptides sequences and acronyms are listed in Figure 3.3d.

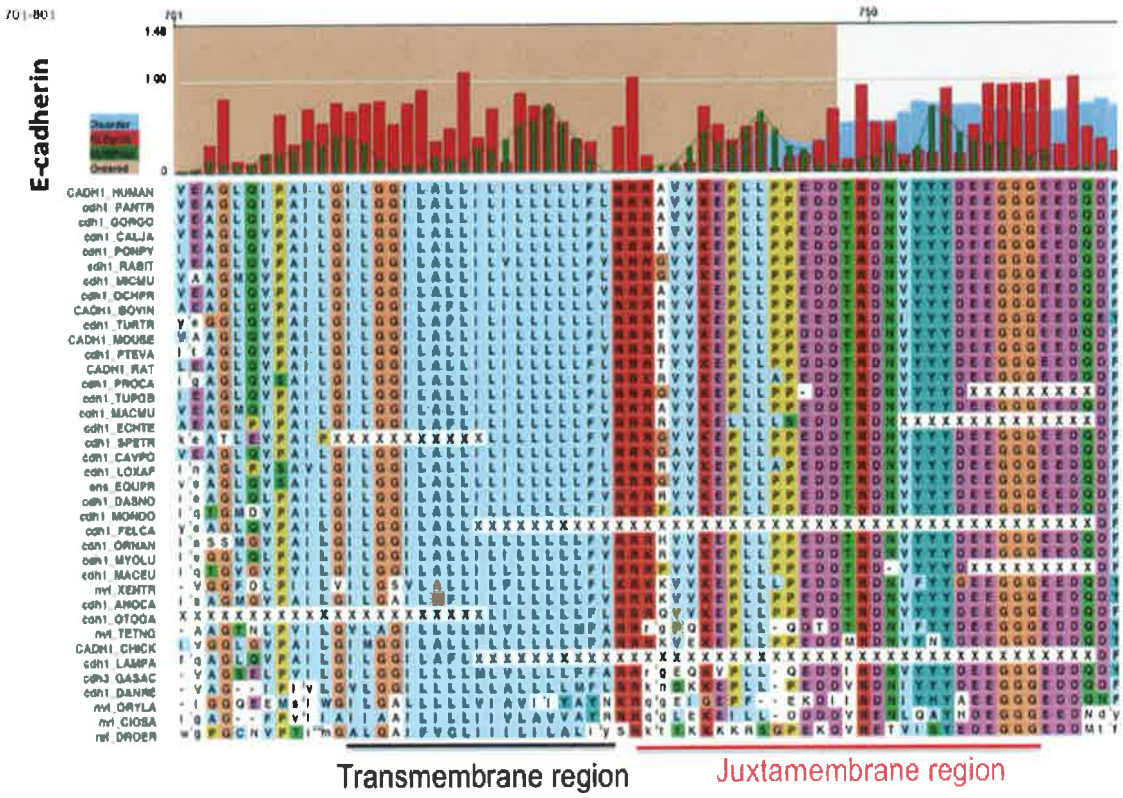


Figure 3.1 SLIM prediction analysis of the JMD of human E-Cadherin. The relative local conservation (RLC) (red bars), disorder region (blue) and SLIM prediction (green bars) are shown on the top graph. The alignment of the transmembrane region and JMD of E-Cadherin (Cadherin-1) with its orthologous proteins is shown on the bottom of the alignment.

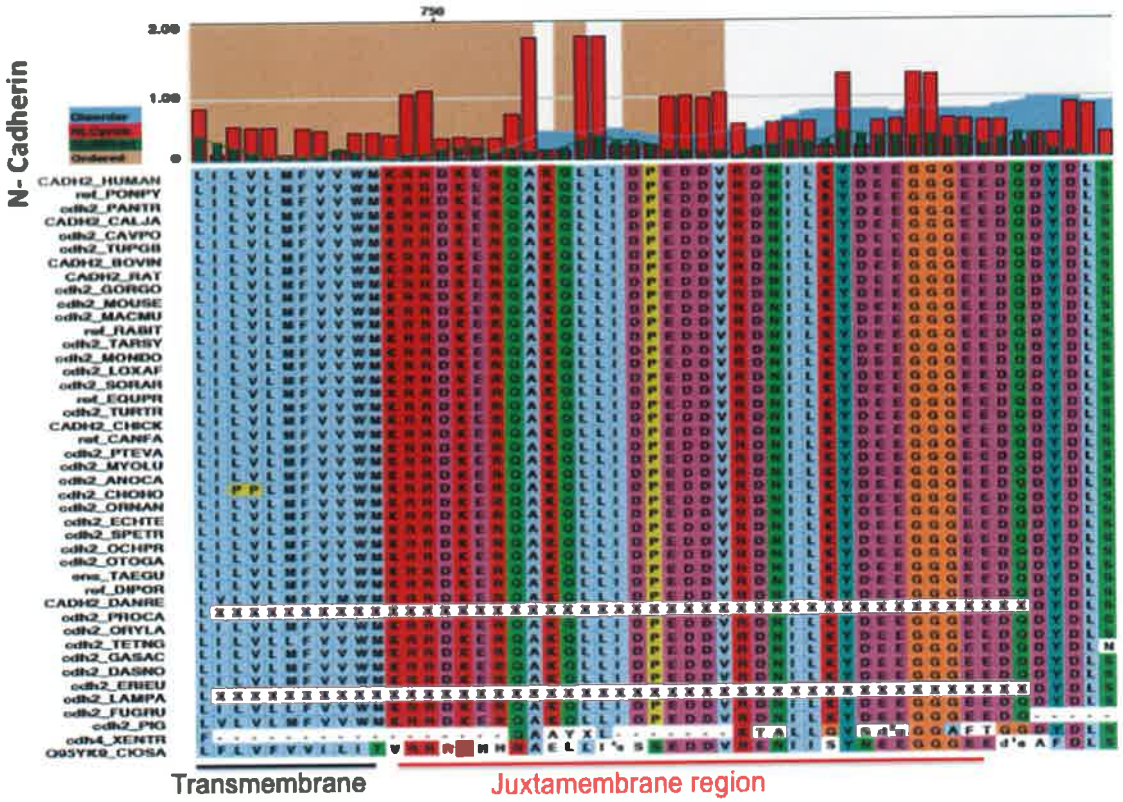
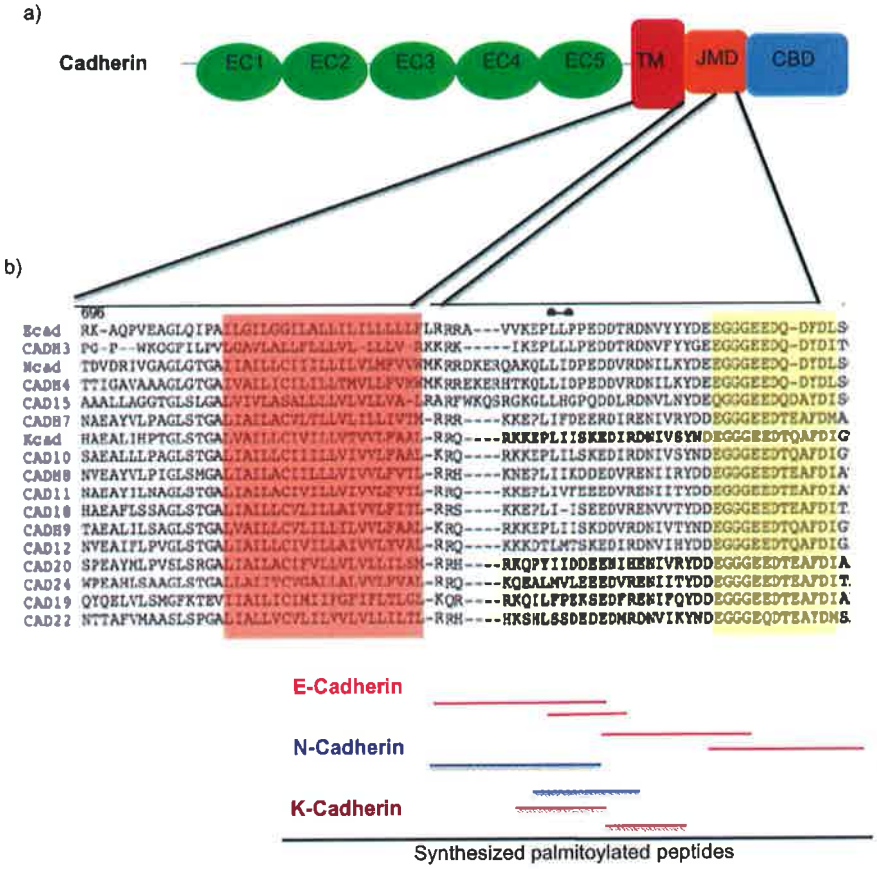


Figure 3.2 SLIM prediction analysis of the JMD of human N-Cadherin. The relative local conservation (RLC) (red bars), disorder region (blue) and SLIM prediction (green bars) are shown on the top graph. The alignment of the transmembrane and JMD of N-Cadherin (Cadherin-2) with its orthologous protein is shown on the bottom of the alignment.



d)

Protein	Peptide Sequence	Acronym
E-Cadherin	RRRAVVKEPLLP	E-Cad 1
E-Cadherin	KEPLLPPEDDT	E-Cad 2
E-Cadherin	EDDTRDNYYYDEE	E-Cad 3
E-Cadherin	DEEGGGEEDQDFDL	E-Cad 4
N-Cadherin	KQLLIDPEDDV	N-Cad 1
N-Cadherin	KRRDKERQAKQLLIDP	N-Cad 2
K-Cadherin	KKEPLIISKE	K-Cad 1
K-Cadherin	RRQRKKEPLIISKE	K-Cad 2

Figure 3.3 a) Graphical representation of the domain structure of human classical cadherins containing 5 extracellular (green) cadherin-like domains (C1-C5), transmembrane region (TM; red), juxtamembrane domain (JMD; orange) and catenin binding region (CBD; blue). b) A zoomed-in image of the multiple sequence alignment of the JMD of different human cadherins. The TM region and P120-catenin binding regions are highlighted orange and yellow color, respectively. c) Peptide sequences designed from E- N- and K-Cadherins are indicated below their position in the alignment. d) List of peptide sequences and acronyms are listed in table. Note: Numerical qualifiers indicate different SLIM sequences from the parent cadherin E-Cadherin, N-Cadherin or K-Cadherin.

3.2.2 Characterization of assay parameters in human platelets

Thrombin receptor activating peptide (TRAP) was chosen as the platelet agonist for the entire study. TRAP is a 6 amino acid (SFLLRN) peptide that binds to the second extracellular loop of the protease activated receptor 1 (PAR1) and triggers platelet activation (Vu et al., 1991). It was chosen for use because it represents a major platelet activator, thrombin. However, thrombin can be problematic to work with due to batch variations and its instability in storage. In contrast, TRAP is stable and reliable and produces consistent responses. To identify the ideal concentration of TRAP to use in this study, a dose response experiment was performed to assess the effect of increasing concentrations of TRAP on platelet ATP secretion. TRAP induced a concentration dependent response that was saturated at 16μM (Figure 3.4). A concentration of 4μM was chosen for all further studies as it induced a response that equates to approximately 80% of the maximal response. By using this sub-maximal dose, it is possible to identify both inhibitory and activatory responses to agents in our assays.

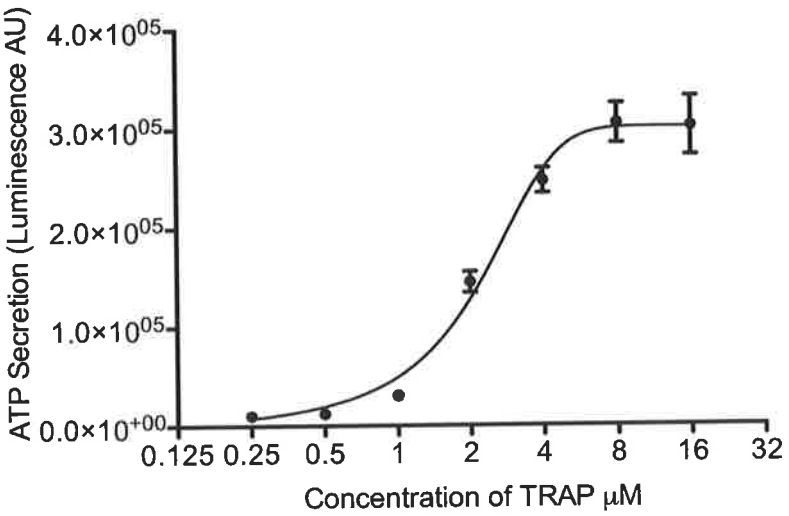


Figure 3.4 Dose-response curve for TRAP in platelet ATP secretion. Washed platelets were incubated with various concentrations of TRAP (from 0.25 to 16μM) at 37°C for 3 minutes. The secreted ATP from platelets was expressed as luminescence arbitrary units (AU). Data represents mean ±standard error mean (SEM) of N= 4 individual donors.

3.2.3 Platelet ATP secretion assay is an ideal method to assess the biological activity of palmitoylated peptides.

Rapid analysis of the bioactive effect of peptides on platelets required a reliable high-throughput assay of platelet function. Moran *et al.* previously published an assay of platelet function based on platelet aggregation (Moran et al., 2006). However, this assay is highly variable from donor to donor, making it difficult to use to assess small differences in peptide bioactivity. To help identify the optimal assay for this study, four peptides were used that had been previously characterized and published by others (Aylward et al., 2006, Covic et al., 2002b) (Table 3.1) in the platelet ATP scretion assay. The four peptides were from integrin αIIb (Aylward et al., 2006) and PAR1 (Covic et al., 2002a) (Table 3.1). Integrin peptides ITGα2b-FF and control of ITGα2b-FF (ITGα2b-AA), are known to be a platelet agonist and antagonist, respectively in platelet

aggregation assays (Aylward et al., 2006). These peptides exhibited the same effect in the high-throughput platelet ATP secretion assay. Specifically, the ITGα2b-FF (Pal-KVGFFKR) was found to be an agonist and could promote platelet ATP secretion in the absence of a platelet agonist (Figure 3.5 grey bars). In contrast, ITGα2b-AA (Pal KVGAAKR) on its own, fails to induce any response (Figure 3.5 grey bars). However, in the presence of a known agonist TRAP (4μM), the ITGα2b-AA peptide abolished platelet secretion response from platelets (Figure 3.5 black bars). Similarily, the peptides PAR1-1 and PAR1-2 (Table 3.1) were previously characterized as platelet antagonist and agonist, respectively (Figure 3.5) (Covic et al., 2002a). Therefore, the activity of PAR1-1 and 2 peptides was analysed using the platelet ATP secretion assay. PAR1-1 alone did not exert any effect on platelet function (Figure 3.5 grey bars) while the PAR1-2 peptide at 50μM induced platelet activation, this result is consistant with previous report where they observed platelet aggregation at 10μM (Covic et al., 2002a) (Figure 3.5 grey bars). However, in contrast to the previous study (Covic et al., 2002a), only 30 to 40% of the TRAP induced platelet ATP secretion response was inhibited in the presence of 50μM PAR1-1 peptide (Figure 3.5 black bars) but significant inhbition of platelet aggeregation was observed at 5μM in previous study indicating that the response to PAR1-1 can differ in different assays (Covic et al., 2002a). In addition, palmitic acid and amide (PAL-NH2), used as a control in this study, failed to induce platelet ATP secretion on its own (Figure 3.5 grey bars) and it did not interfere with TRAP induced platelet ATP secretion (Figure 3.5 black bars).

Together, these observations suggest that the platelet ATP secretion assay (Sun et al., 2001, Edwards et al., 2007) is a reliable high throughput assay as it effectively reproduced the previously identified peptides responses as described by Aylward *et al.* and Covic *et al.* (Covic et al., 2002a, Aylward et al., 2006). Therefore, this assay has been chosen for analysis of all the peptides in this study.

Table 3.1. Peptide sequences of integrin and PAR (Covic et al., 2002a, Aylward et al., 2006) .

Protein	Peptide Sequence	Acronym
Integrin α2b	KVGFFKR	ITGα2b-FF
Control of ITGα2b-FF	KVGAAKR	ITGα2b-AA
PAR1	RCLSSAVANRS	PAR1-1
PAR1	AVANRSKKSRALF	PAR1-2

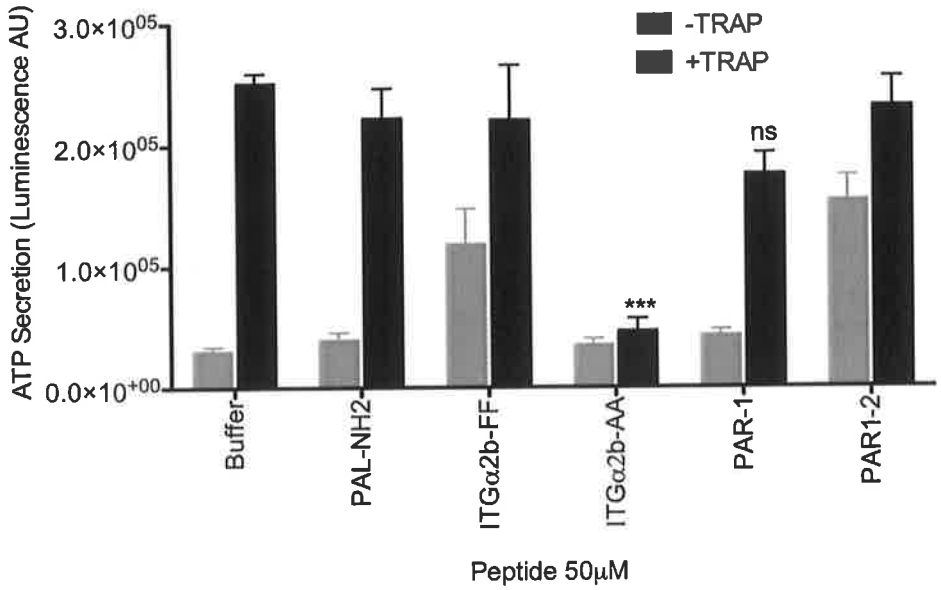


Figure 3.5 Platelet ATP secretion in response to Integrin-derived and PAR peptides in the presence and the absence of TRAP. Washed platelets were treated with 50μM of each peptide for 12 minutes at 37°C followed by activation with TRAP (4μM) for 3 minutes. Grey bars indicate platelets ATP secretion by platelets in response to peptides/buffer and black bars indicate platelet ATP secretion in the presence of peptides following stimulation with TRAP. Data is expressed as the amount of ATP secretion measured by luminescence arbitrary units (AU). Data represents mean ±SEM of N=6 individual donors. ***P<0.0001; significance was obtained using One-way ANOVA by comparing the response of platelets to TRAP in the absence of any peptide.

3.2.4 Effect of novel cadherin-derived peptides on TRAP induced platelet secretion

To investigate the effects of E- and N-Cadherin peptides on the platelet response to TRAP, human washed platelets were incubated for 12 minutes at 37°C with 50μM of peptide or buffer prior to addition of TRAP (4μM). Platelet ATP secretion was measured after 3 minutes incubation with TRAP. Peptides from K-Cadherin are known to attenuate the TRAP-induced platelet ATP secretion (Edwards et al., 2007). Therefore, K-Cadherin peptides were used as positive control (Figure 3.6). N-Cad 1 and N-Cad 2 peptides has the most profound inhibitory effect. They

inhibited TRAP-induced ATP secretion by 80% and 95%, respectively (black bars; Figure 3.6). The peptides derived from E-Cadherin also significantly inhibited TRAP-induced ATP secretion by 50% to 70% (Figure 3.6). This is indicated that E- and N-Cadherin peptides were able to modulate platelet function. Thus, the peptides are acting as antagonists of platelet function. The activity of peptides, in the absence of any platelet agonist, was also investigated to examine whether E- and N-Cadherin peptides can act as agonist-peptides. For most peptides, there was no ATP secretion observed. However, E-Cad 1 induced 30% of platelet ATP secretion ($P<0.0001$) compared to TRAP (100%), suggesting that this peptide could have an activating effect on platelets (Figure 3.6). In parallel experiments, the peptide linker Pal-Ttds-NH2 (50μM) and Dimethyl sulfoxide (DMSO) (vehicle control) did not show any substantial effect on TRAP induced platelet ATP secretion (Figure 3.6) suggesting that the observed effects of the E and N-Cadherin peptides were not caused non-specifically by the palmitic acid.

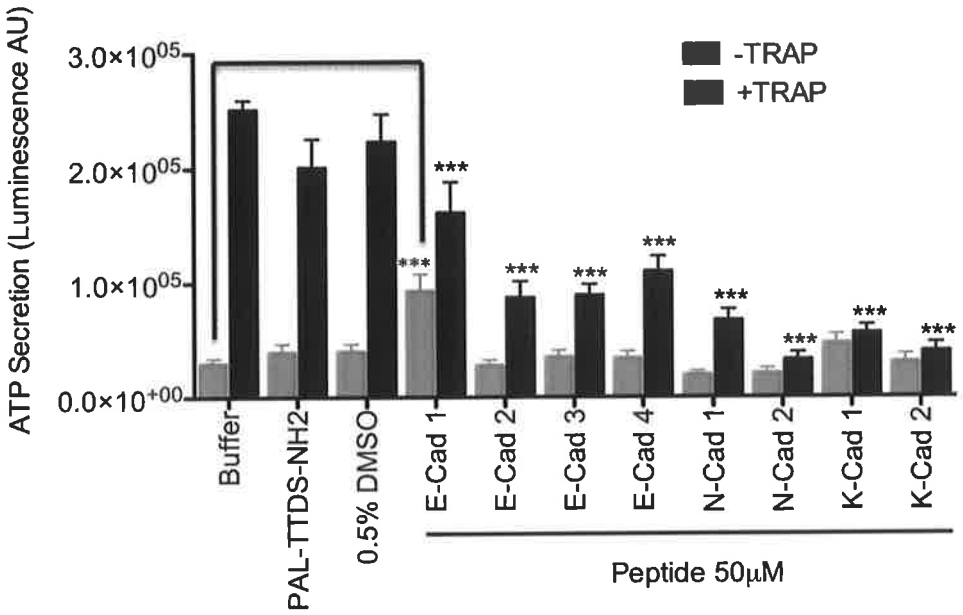


Figure 3.6 Platelet responses to novel cadherin peptides. 50μM of Cadherin-derived peptides were incubated with washed platelets at 37°C for 12 minutes followed by activation of platelets with TRAP (4μM). Grey bars indicate platelet ATP secretion in the presence of peptide/buffer and black bars indicates platelet ATP secretion in the presence of peptides following stimulation with TRAP. Data shown is mean ±SEM of N=6 independent donors and are expressed as detailed above (see figure legend Fig.3.5). ***P<0.0001 represents significance calculated using One-way ANOVA. Significance was obtained by comparing the response of platelets to TRAP in the absence of any peptide.

3.2.5 Potency of cadherin-derived peptides

Most of the novel cadherin-derived peptides modulated platelet dense-granule secretion induced by TRAP (Figure 3.6). Therefore, the potency of selected peptides in ablating platelet function at low doses was next assessed using different concentrations from 0.38μM to 50μM. Specifically, three peptides were chosen to explore the effect of low doses of E-Cad 2 and N-Cad 2 because of their potent inhibitory properties (Figure 3.6) and the E-Cad 1 peptide due to its activating (P<0.0001) and inhibitory nature (Figure 3.6). Dose response curves showed that 25μM is the minimum concentration of each peptide that can significantly inhibit TRAP induced platelet ATP secretion (Figure 3.7 black

lines). E-Cad 2 and N-Cad 2 peptides alone did not exert any effect on platelet response (Figure 3.7b and c grey lines). Interestingly, a trend towards a pro-activating effect was observed following platelet incubation with high doses of E-Cad 1 peptide (Figure 3.7a grey line). The activity of cadherin-derived peptides were further analysed in platelet aggregation (gold standard platelet functional assay). Importantly, as evident from Figure 3.7 while the peptides tested (E-Cad 1,2 and N-Cad 2) failed to induce platelet aggregation at a concentration of 50μM (data not shown), they effectively inhibited platelet aggregation at 25 and 50μM (Figure 3.8). This data confirmed and reinforced the results observed in the platelet ATP secretion assay (Figure 3.7). All together, these results suggest that cadherin-derived peptides are able to inhibit both platelet dense granule secretion and aggregation in a dose dependent manner. The minimum concentration of peptide required to abolish the TRAP induced platelet activation was found to be 25μM .

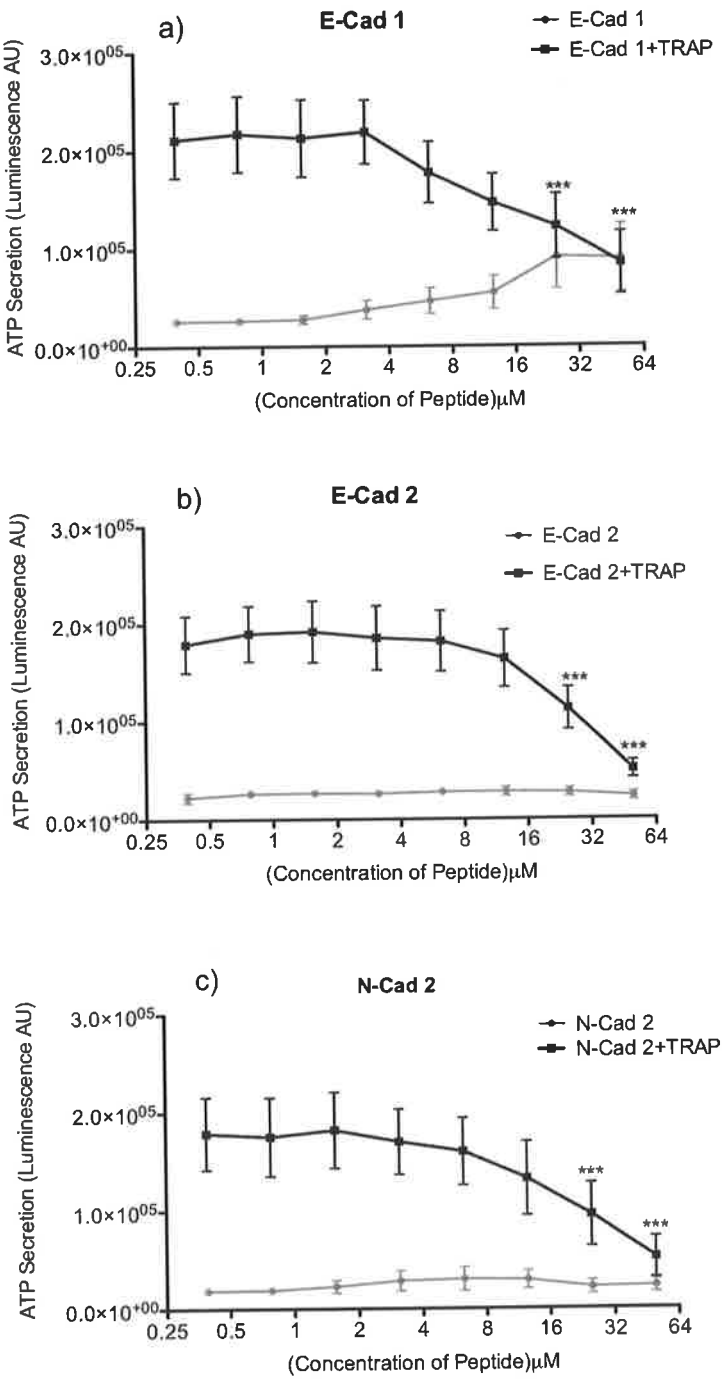


Figure 3.7 Dose dependent inhibition of TRAP induced platelet ATP secretion with cadherin-derived peptides. Different concentrations (0.39,0.78,1.56, 3.13, 6.25, 12.5, 25 and 50μM) of cadherin-derived peptides were incubated with platelets at 37°C for 12 minutes. The amount of ATP released by platelet dense granules was measured following 3 minutes stimulation with TRAP (4μM). Grey lines indicate resting platelet response in the presence of the reported peptide while black lines show platelet response in the presence of the peptide and following stimulation with TRAP. Data is expressed as detailed in figure legend Figure 3.5. Data represent mean ±SEM of N=5 independent donors. ***P<0.0001 represents significance calculated using One-way ANOVA. Significance was obtained by comparing the response of platelets to TRAP in the absence of any peptide.

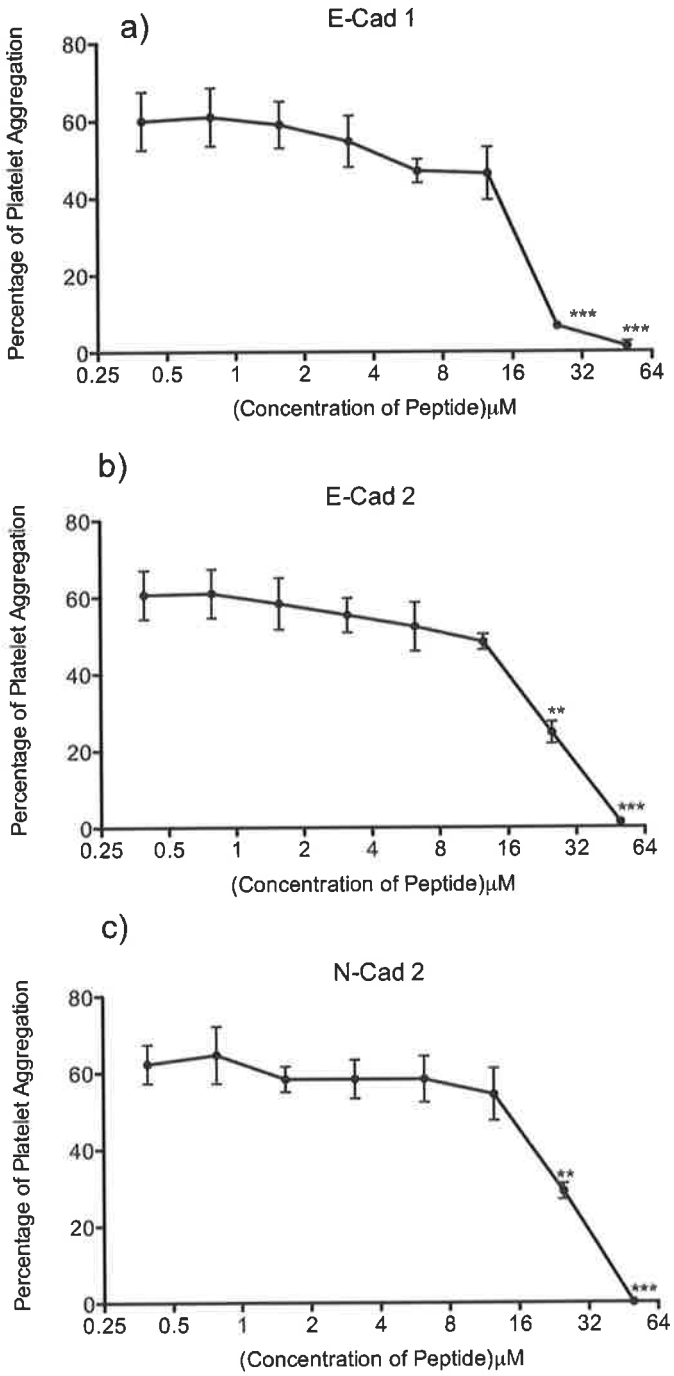


Figure 3.8 Effect of various doses of cadherin-derived peptides on TRAP induced platelet aggregation. Different concentrations (0.39, 0.78, 1.56, 3.13, 6.25,12.5, 25 and 50μM) of) cadherin-derived peptides were incubated with platelets at 37°C for 5 minutes under stirring conditions (1100rpm) and aggregation was monitored following addition of TRAP (4μM). Data expressed as percentage of platelet aggregation and error bars represent mean ±SEM of N=3 independent donors. **P<0.001 and ***P<0.0001 indicate significance caluculated using One-way ANOVA analysis. Significance was obtained by comparing the response of platelets to TRAP in the absence of any peptide.

3.2.6 Sequence specificity of cadherin-derived peptides

Given the ability of the cadherin-derived peptides employed in this study to inhibit TRAP induced platelet responses, it was necessary to determine their sequence specificity. To address the issue of peptide specificity, a number of control peptides were designed to E-Cad 1, E-Cad 2 and N-Cad 2 (parent peptides). The control peptides are derived from parent the peptide sequence with some modifications such as amino acid replacement and scrambling. The design of control peptides is not a trivial task as many potential control peptides can be created to attempt to control for peptide sequences. In the simplest form, it would be possible to generate 10-factorial (10! = 3,628,800) possible scrambled peptides from a single parent sequence composed of 10 different amino acids. However, different peptides may have different solubility or charge distribution that may explain their effects. Moreover, the effect of a given peptide may depend on its charge and/or hydrophobicity rather than its precise sequence. Clearly it was not be possible to assess all possible control peptide sequences. Instead 5 different control peptides for each parent peptide (E-Cad 1, 2 and N-Cad 2) were designed using the control peptide design program (Table 3.2) (http.//bioware.ucd.ie/~cyclops/Fergal/tags/PepControls).

- **Scramoncharge**: The non charged amino-acid (AA) residues within the sequence were scrambled and charged AAs were replaced with different AAs containing the same charge. For

example, scramoncharge peptide for RRRAVVKEPLLP could be KKKPLARDVVPL.

- **Reverse charge:** Positvely charged AAs within the sequence were replaced with negative AAs and vice-versa. Other AAs remain the same. Thus a reverse charge peptide for RRRAVVKEPLLP would be EEEAVVEKPLLP
- **Randomly scrambled:** AAs within the peptide sequence were randomly scrambled. Thus a control for RRRAVVKEPLLP could be PVRERVLARLKP.
- **Di-reverse:** Reverse the order of the AAs in pairs from C-terminus to N-terminus. Thus a control for RRRAVVKEPLLP would be LPPLKEVVRARR.
- **Replacement of AAs:** Highly conserved residues in the peptide sequence (in orthologous proteins) are replaced with different AAs. Thus replaced AA for RRRAVVKEPLLP would be RERAVVKEPLLP.

Table 3.2 List of E-Cad 1, E-Cad 2 an N-Cad 2 control peptide sequences. Red color amino acids (AAs) represents replacement of highly conserved AAs within the sequence (in orthologous proteins) replaced with different AAs.

Peptide Sequence	Acronym
E-Cad 1 Peptide series	
RRRAVVKEPLLP	E-Cad 1
KKKPLARDVVPL	E-Cad 1 Scramoncharge
EEEAVVEKPLLP	E-Cad 1 Reverse charge
PVRERVLARLKP	E-Cad 1 Randomly scrambled
LPPLKEVVRARR	E-Cad-1 Di-reverse
RERAVVKEPLLP	E-Cad-1 R to E
RRRAVVKEPL**A**P	E-Cad-1 L to A
E-Cad 2 Peptide series	
KEPLLPPEDDT	E-Cad 2
RDPTLLPDEEP	E-Cad 2 Scramonchrage
EKPLLPPKKKT	E-Cad 2 Reverse charge
DPDLELTKPDE	E-Cad 2 Randomly scrambled
DTEDPPLLEPK	E-Cad 2 Di-reverse
KEPLLPPED**K**T	E-Cad 2 D to K
KEPLLPP**K**DDT	E-Cad 2 E to K
KEPL**A**PPEDDT	E-Cad 2 L to A
N-Cad 2 Peptide series	
KRRDKERQAKQLLIDP	N-Cad 2
RKKERDKILRPQALEQ	N-Cad 2 Scramoncharge
EEEKEKEQAEQLLIKP	N-Cad 2 Reverse charge
LDKQRPDRIKKQDERL	N-Cad 2 Randomly scrambled
DPLIQLAKRQKERDKR	N-Cad 2 Di-reverse
K**E**RDKERQAKQLLIDP	N-Cad 2 R to E
KRRDKERQAKQ**A**LIDP	N-Cad 2 L to A

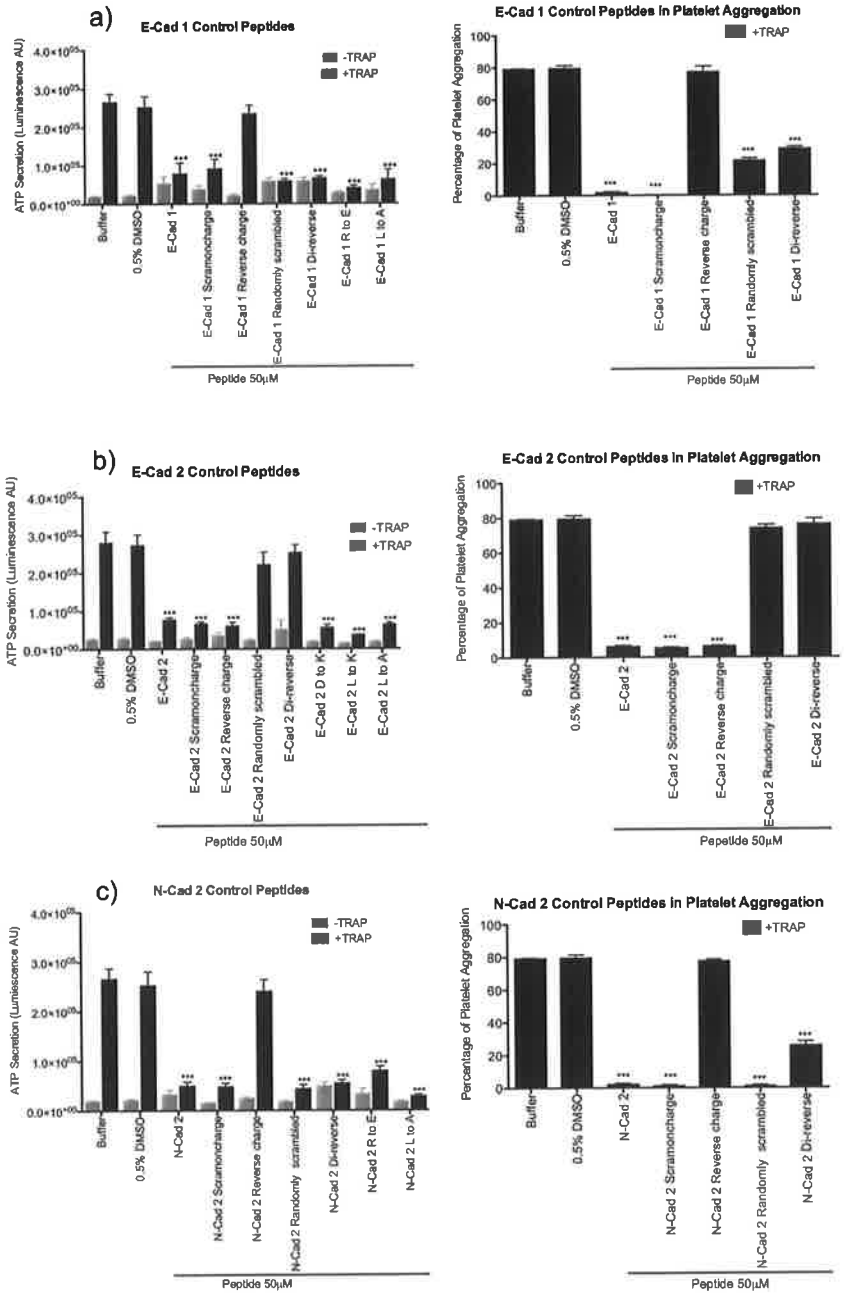


Figure 3.9 Effect of cadherin-derived control peptides on platelet ATP secretion and aggregation induced by TRAP. Washed platelets were treated with 50µM of different control peptides of a) E-Cad 1, b) E-Cad 2 and c) N-Cad 2 at 37°C for 12 minutes in ATP secretion assays (left panel) or for 5 minutes in aggregation assays (right panel) followed by activation of platelets with TRAP (4µM). Grey bars indicate the platelet ATP secretion in the presence of peptide/buffer alone and black bars indicate platelet ATP secretion following stimulation with TRAP. For platelet aggregation assays, all data represents the response observed following TRAP activation for 3 minutes. Error bars indicate mean ±SEM of N=6 individual donors for ATP secretion and N=3 for aggregation. ***P<0.0001 is an indicator of statistical significance as determined using One-way ANOVA. Significance was obtained by comparing the response of platelets to TRAP in the absence of any peptide in both ATP secretion and aggregation assays.

The effects of control peptides were investigated using ATP secretion and aggregation assays. The concentration of peptide was maintained at 50μM in these assays (Figure 3.9). Surprisigly, most of the E-Cad 1, E-Cad 2 and N-Cad 2 control peptides inhibited TRAP induced platelet response. Thus it appears that the sequence of our cadherin-derived peptides is not critical for their actions. For the E-Cad 1 peptide series, the randomly charged peptide inhibited secretion and aggregation in a similar manner to the parent peptide (Figure 3.9a). However, the reverse charge peptide was inactive, suggesting that peptide charge, or charge-distribution may be responsible for the observed effects to date with this peptide (Figure 3.9a). In contrast, the randomly scrambled E-Cad 2 peptide was devoid of inhibitory actions, but the reverse charged peptide was equally as potent as the parent peptide (Figure 3.9b). For this peptide however, scrambling of the uncharged residues and reverse charge peptides preserved the activity, this observation making it difficult to speculate on what is critical for peptide activity. Finally, for the N-Cad 2 peptide series, the randomly scrambled and di-reverse peptides also showed inhibitory activity similar to the parent peptide (Figure 3.9c). However, the reverse charge peptide was inactive. This is suggests charges are important for activity (Figure 3.9c). In addition, the conserved AA replaced peptides (example "E Cad-1 R to E or L to A" Table 3.2) had identical effects when compared to the parent peptides on platelet function in both assays (Figure 3.9). This observation suggests that conserverd amino acids (AAs) within the peptides sequence (in orthologous proteins) are not crucial for biological activity in our assays.

Together these observations raise the critical question of the specificity of action for all peptides in this study.

For all of this data it is noteworthy that the agregation responses exactly mirror those observed in the ADP secretion assay. This supports our decision to utilize the ATP-secretion assay as the main assay in this study. It is more rapid and easier to perform than the platelet aggregation assay. It also requires lower volumes of washed platelets allowing a greater number of experimental variables to be assessed per donated blood sample.

3.2.7 Mapping the inhibitory activity of the amino acid residues within the peptide sequence

In parallel to sequence specificity studies, key amino acids (AAs) responsible for the profound inhibitory effect of the cadherin-derived peptides (E-Cad 1 E-Cad 2 and N-Cad 2) on platelet function was examined by using truncated peptides. Truncated peptides were synthesized by systematic deletion of each amino acid (AA) from N- and C-terminus of E-Cad 1, E-Cad 2 and N-Cad 2 (parent peptides). The effect of the truncated peptides was assessed in the platelet ATP secretion assay. None of the truncated peptides induce the platelet activation. In contrast, most of the peptides inhibited TRAP-induced platelet ATP secretion (Figure 3.10). However, E-Cad 1 peptides (RRRAVVKEPLLP) incrementally lost their inhibitory activity by sequential deletion of positively charged amino acids (AAs) from the N-terminus, but

deletion of C-terminus AAs did not influence the inhibitory activity (Figure 3.10a). Similarly, N-Cad 2 peptides incrementally lost activity following deletion of N-terminal AAs (Figure 3.10c). In contrast, most of E-Cad 2 (KEPLLPPEDDT) truncated peptides did not show altered activity compared to the parent peptide (Figure 3.10b). In addition, peptide with the short motif KEPLLP peptides exhibited most potent activity in the E-Cad 2 truncated peptide series (Figure 3.10b). Deletion of C-terminus residues in N-Cad 2 peptide (KRRDKERQAKQLLIDP) had no effect on the inhibitory activity suggesting that only the N-terminal sequence is bioactive. However, deletion of predominantly charged AAs from the N-terminus resulted in a loss of peptide inhibition activity (Figure 3.10c). Together, these truncation results suggested that positive charged AAs in cadherin peptide sequences are crucial for peptide bioactivity and these results also highlighted KEPLLP peptides as a potent platelet function inhibitor.

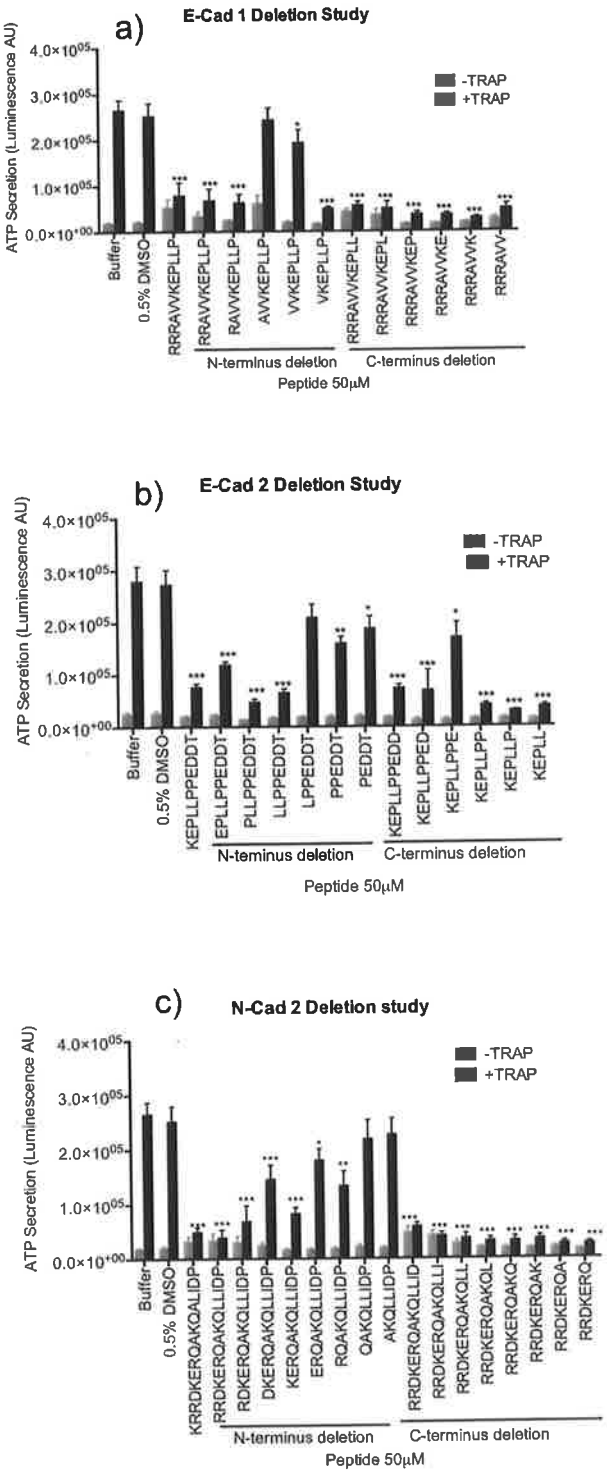


Figure 3.10 Effect of cadherin-derived truncated (deletion) peptides on TRAP induced platelet activation. Cadherin truncated peptides (50μM) were incubated with platelets for 12 minutes at 37°C followed by activation of platelets with TRAP (4μM). The platelets response to peptides was measured in the absence (grey bars) and presence (black bars) of TRAP. Error bars indicate mean ±SEM. Statistical analysis was performed using One-way ANOVA of N=6 individual donors, *P<0.05, **P<0.001 and ***P<0.0001. Significance was obtained by comparing the response of platelets to TRAP in the absence of any peptide.

3.2.8 Focus on the short motif KEPLLP

The Cadherin control peptide and truncated peptide study suggested that two separate features regulated peptide activity. (1) Peptides with positive charges on the N-terminus, adjacent to the palmitate moiety, are potent inhibitors and (2) The E-Cad 2 sequence KEPLLPPEDDT appeared to independently possess sequence-specific inhibitory activity (Figure 3.9). In addition this study identified a motif of 6 AAs (KEPLLP) (Figure 3.10) as a potent inhibitor of platelet response to TRAP.

Previous studies have identified that the KEPLLP motif as a dynamic binding domain for P120-catenin in E-Cadherin. In particular, the LL di-leucine motif appears to be crucial for the interaction of JMD of E-Cadherin with P120-catenin (Ishiyama et al., 2010).

Based on these observations it was hypothesized that the KEPLLP peptide might play a specific role in platelet function and that the LL motif within the peptide might be crucial for bioactivity. This hypothesis was tested by replacement of each amino acid with alanine (Alanine scanning) to identify the key residues within KEPLLP (Table 3.3). In addition, sequence specificity of the KEPLLP peptide was also analyzed using control peptides (Table 3.3). The effect of KEPLLP peptides, and their corresponding control peptides, was analyzed by platelet ATP secretion assay at various concentrations (0.8, 3, 12.5 and 50μM) in the presence of TRAP (4μM) as platelet agonist (Figure 3.11). The peptides alone did

not induce platelet secretion (data not shown). However, they inhibited TRAP induced ATP secretion in a dose dependent manner (Figure 3.11).

Table 3.3 List of KEPLLP Alanine-scanning peptides (a) and control peptides (b). Red amino acids (AAs) indicate replaced AAs within the sequence.

a)

Peptide Sequence
KEPLLP
KEPLLA
KEPLAP
KEPALP
KEALLP
KAPLLP
AEPLLP

b)

Peptide Sequence	Acronym
REPLLP	K to R
EEPLLP	K to E
KDPLLP	E to D
KEALLA	P to A
KEPAAP	LL to AA
KEPVVP	LL to VV
EKPLLP	Reverse KE
PLPLEK	Randomly Scrambled
KLEPPL	Randomly Scrambled
PLLPKE	Randomly Scrambled

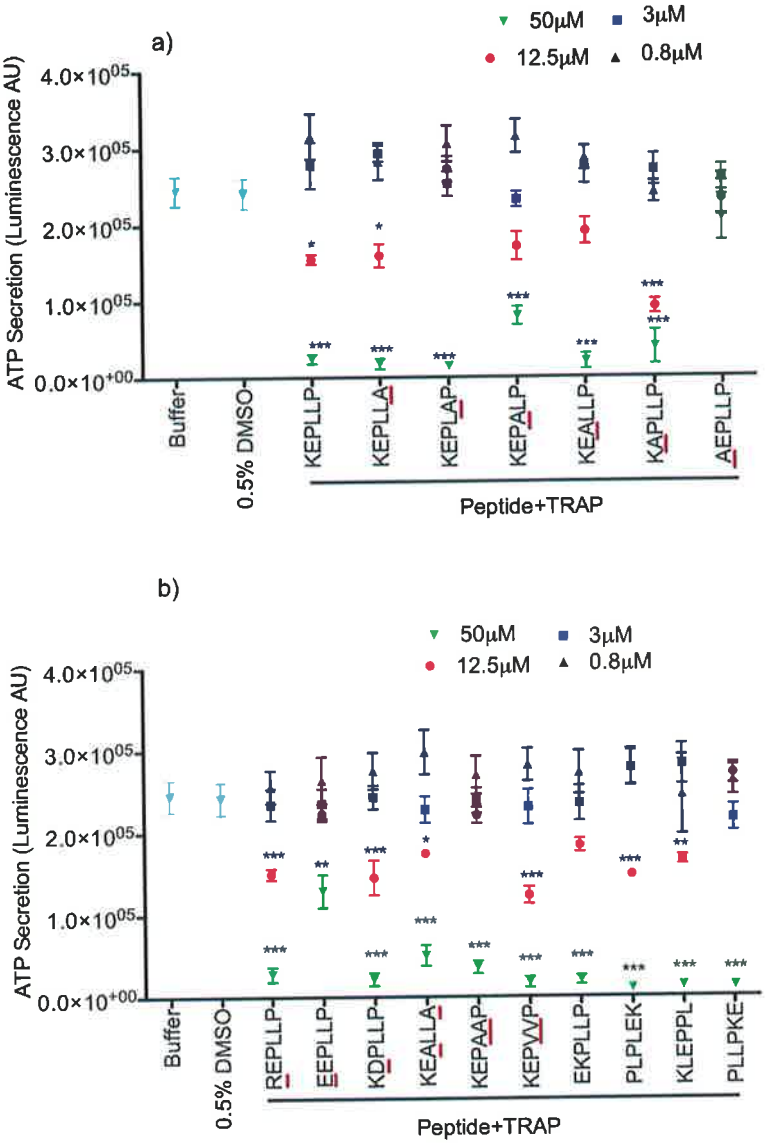


Figure 3.11 Effect of KEPLLP control peptides on platelet ATP secretion. Washed platelets were treated with various concentrations (0.8, 3, 12.5 and 50μM) of peptides for 12 minutes at 37°C followed by activation of platelets with TRAP (4μM) for 3 minutes. Data is expressed as the amount of ATP secretion measured by luminiscence AU. a) Alanine-scanning peptides. b) Control peptides. Error bars represent mean ±SEM of N=4 individual donors. *P<0.05, **P<0.001 and ***P<0.0001 represents significance, calculated using One-way ANOVA. Significance was obtained by comparing the response of platelets to TRAP in the absence of any peptide.

A dose response analysis of the effects of alanine-scanning and control peptides on platelet function suggested that none of the peptides had effects at low doses (0.8 and 3μM) (Figure 3.11). At 50μM most of the KEPLLP peptides inhibited platelet function in a non-specific manner

probably due to the high concentration of peptides (Figure 3.11). However, a particular set of peptides at 12.5μM inhibited the platelet response to TRAP. Therefore, 12.5μM is the ideal concentration of peptide to use in this assay as this is the lowest concentration with biological activity and therefore will enable interpretation of the results with the least potential for non-specific effects.

The replacement of leucine at position 4 and 5 (KEPALP and KEPLAP) caused loss of inhibitory activity. Similarly replacement of the LL motif with two alanines (KEPAAP) also caused loss of activity (Figure 3.11). However, replacement of the LL motif with another hydrophobic amino acid namely valine (V), recovered (KEPVVP) the inhibitory activity (Figure 3.11b). These results suggested that the LL motif is not crucial for biological activity but hydrophobic residues are required for bioactivity. In addition, analysis of KEPLLP peptides also suggested that PLLP motif could play role in anti-platelet activity. There are seven peptides (KEPLLP, KAPLLP, REPLLP, KDPLLP, EEPLLP, EKPLLP and AEPLLP), which contains intact PLLP motifs. Of these, some peptide are potent as KEPLLP peptide and while others are not (Figure 3.11). The bioactivity of all of these peptides is associated with positive AA (K or R) at the first position but peptides with PLLP without positive charge at first position lose their activity. Therefore PLLP itself is not important for activity but a positive charge at position 1 is important. In contrast, there are eight peptides, which have a positively charged amino acid at position 1 with no PLLP (KEPLLA, KEPLAP, KEPALP, KEALLA, KEPAAP, KEPVVP,

KEALLP and KLEPPL). Of these, some peptides retain the anti-platelet activity and some lose the activity. This is clearly suggests that, simply a positive charge at position 1 is not sufficient for activity. However, a combination of positive charge with hydrophobic residues oriented by proline seems to have consistent inhibitory activity.

3.2.9 Toxicity of cadherin peptides

According to the results showed in the Figures 3.6 to 3.11, the majority of the cadherin-derived (long and short) peptides, including controls, used in this study greatly impaired platelet function. The mechanism of action of these peptides is still unknown. To further investigate whether the inhibitory activity observed was a consequence of peptide activity or associated with toxic properties, a lactate dehydrogenase (LDH) assay was employed to assess the toxicity of peptides. Initially a small selection of peptides was tested to establish if this assay was a valid assay for the assessment of toxicity in platelets (Figure 3.12).

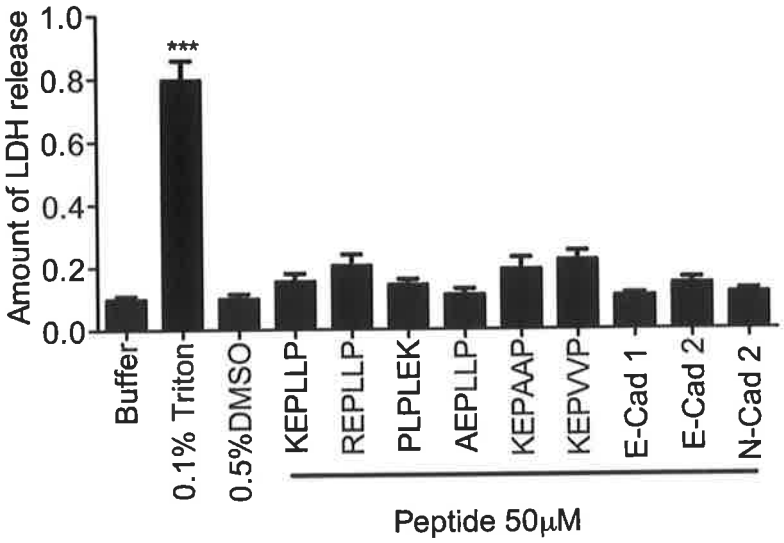


Figure 3.12 LDH-Toxicity analysis of Cadherin-derived peptides. Washed platelets were treated with 50μM of peptides at 37°C for 15 minutes under aggitation (300rpm). After treatment, samples were cenrifuged and 50μl of the supernant was added to 100μl of LDH reagent in a clear 96-well plate. The samples were incubated for 20 minutes in the dark and absorbance was measured using Perkin-Elmer spectrophotometer at 490nm wavelength. Buffer treated platelets were used as negative control and 0.1% Triton 100X treated platelets were used as positive controls. Data represents mean ±SEM of N=4 individual donors, where ***P<0.0001; One-way ANOVA. Significance was obtained by comparing with platelet LDH release in the presence of buffer (negative control).

As evident from Figure 3.12, none of the peptides induced LDH leakage when compared with intact platelets (platelets in buffer). As expected, Lactate dehydrogenase was abundantly released from platelets in the presence of lysis buffer (0.1% Triton100X), used as positive control. These results demonstrate that the inhibitory activity of the peptides is not associated with toxic properties. However, release of LDH from platelets would need substantial damage to the membrane. It is possible that the peptides used in this assay might not cause substantial damage to the platelet membrane but they might cause a lesser toxicity, which cannot be detected by the LDH assay. Therefore, there is a need for an optimal assay, which can detect the partial toxicity. We explored whether a more

sensitive toxicity assay could be used to detect the acute toxicity of these peptides.

The toxicity of cadherin-derived peptides was further analysed in a more sensitive assay developed using red blood cells (RBCs). The membranes of RBC contain approximately equal amounts of lipids and proteins (Nayak et al., 2008). The RBC membrane is more fragile when compared to platelets (Leidl et al., 2008). Due to their fragile membrane, RBC become more prone to damage in low pH, low levels of glucose and high local concentrations of toxic free radicals (Gallagher, 2005). In addition, unlike platelets, RBCs are easily lysed by hypotonic solutions. Whenever RBCs are exposed to toxic substances such as triton they release hemoglobin into the buffer or supernatant because of damage to the cell membrane. Therefore, in this chapter, an assay was set up for detecting whether the peptides could cause damage to the fragile RBC membrane.

Initially the toxicity of all E-Cad 1,2, N-Cad 2 and their control peptides (Table 3.2) was assessed in the RBC lysis assay at 50μM concentration to detect the acute toxicity of peptides. There was no damage to RBCs treated with peptides, as measured by the release of hemoglobin. While 0.1% Triton100X was effective at causing red cell lysis as measured in this assay, none of the peptides (Table 3.2) or their corresponding control peptides induced the toxicity to the RBCs (Figure 3.13). In addition, the vehicle control 0.5% DMSO was not toxic to the RBCs (Figure 3.13).

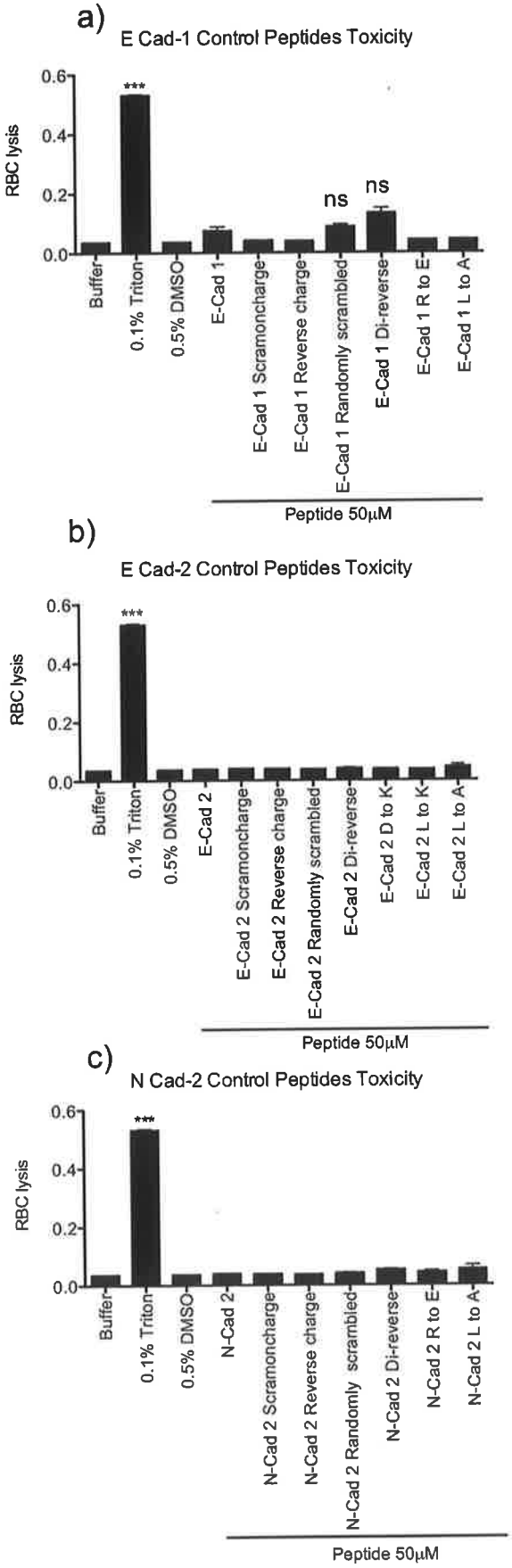


Figure 3.13 Effect of Cadherin-derived peptides and their controls (Table 3.2) in RBC lysis assay. Diluted RBCs (1 in 10) were treated with 50µM of each peptide at 37°C for 15 minutes. Final volume of assay was 300µl. Samples were incubated in thermomixer at 300 rpm. After incubation samples were centrifuged at 1000Xg for 5 minutes and the supernant was collected and analysed at 405nm absorbance for presence of hemoglobin. a) E-Cad 1 control peptides, b) E-Cad 2 control peptides and c) N-Cad 2 control peptides. Data shown is mean ±SEM of N=3 individual expreiments, where *P<0.05, **P<0.001, ***P<0.001 represent statistical significance values determined by One-way ANOVA. Significance was obtained by comparing to RBC treatment with buffer alone (negative control).

In parallel, the effect of truncated or deleted peptides was further assessed in the RBC lysis assay. Analysis of cadherin-derived truncated peptides in the RBC lysis assay showed that most of the peptides did not cause any leakage of hemoglobin from RBC (Figure 3.14) similar to buffer treated RBC (negative control). However, some of the C-terminus deletion peptides were able to cause toxicity to RBCs (Figure 3.14). In particular, from E-Cad 2 C-terminus deletions, the peptide KEPLLP causes a distinct and significant lysis RBC (Figure 3.14b). This is striking that the potent inhibitory effect of KEPLLP discussed above is probably associated with toxicity. In addition, Pal-NH2 did not cause leakage of hemoglobin, indicating that palmitoylation is not toxic to the RBC (Figure 3.14).

To further understand the toxicity of shorter peptides, alanine-scanning and control peptides to KEPLLP (Table 3.3) were also assessed in the RBC lysis assay. These results identified that KEPLLP and some of its alanine scanning peptides were able to cause leakage of hemoglobin (Figure 3.15a). More interestingly, none of the random scrambled peptides of KEPLLP induced the leakage of hemoglobin (Figure 3.15b). Together these results demonstrate that the non-specific effect of cadherin-derived peptides on platelets cannot be explaind by conventional toxicity (LDH) assay. However, a more sensitive assay of toxicity, the RBC assay suggested that some of the cadherin-derived peptides exhibiting non-specific inhibition of platelet function probably due to partial toxicity.

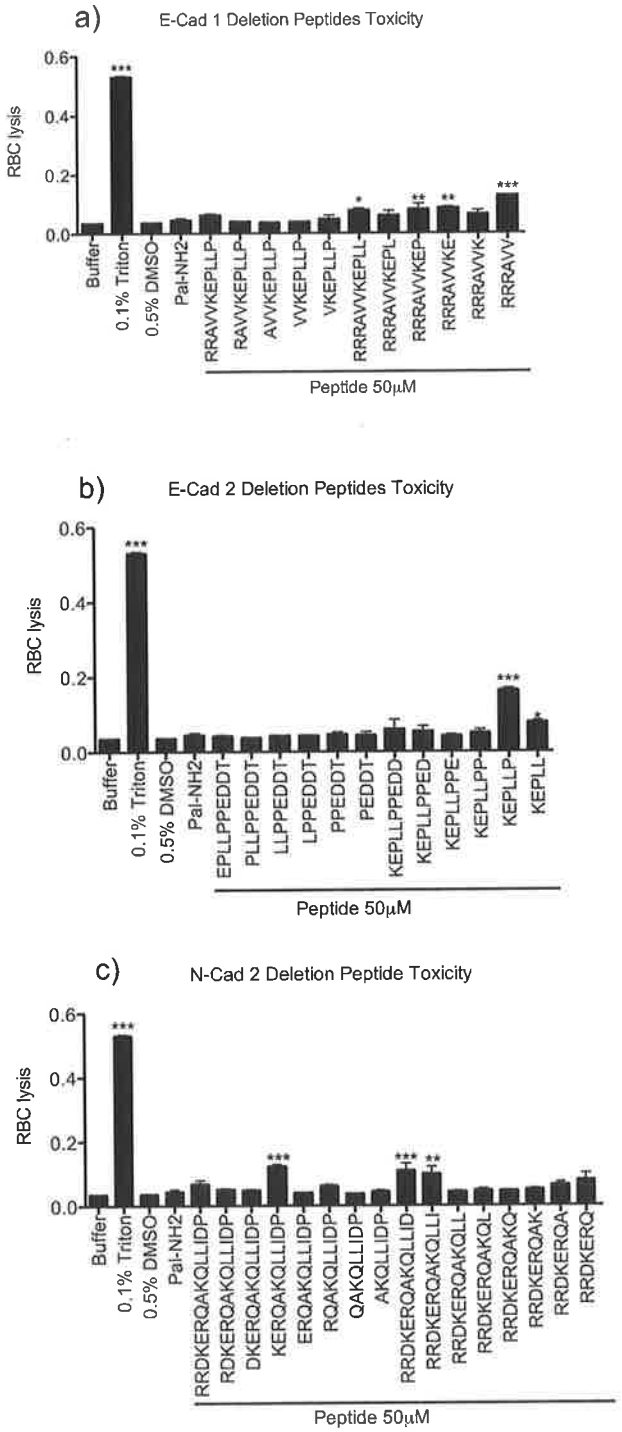


Figure 3.14 RBC lysis assay with Cadherin derived deletion peptides. Diluted RBCs (1 in 10) were treated with 50μM each peptide at 37°C for 15 minutes. The final volume of the assay was 300μl. Samples were incubated in a thermomixer at 300rpm. After incubation, samples were centrifuged at 1000xg for 5 minutes and the supernatant was collected and analysed at 405nm absorbance for presence of hemoglobin. a) E-Cad 1 truncated peptides, b) E-Cad 2 truncated peptides and c) N-Cad 2 truncated peptides. Data represents mean ±SEM of N=3 individual expreiments, where *P<0.05, **P<0.001, ***P<0.001 represent statistical significance values determined by One-way ANOVA. Significance was obtained by comparing with RBC treatment with buffer alone (negative control).

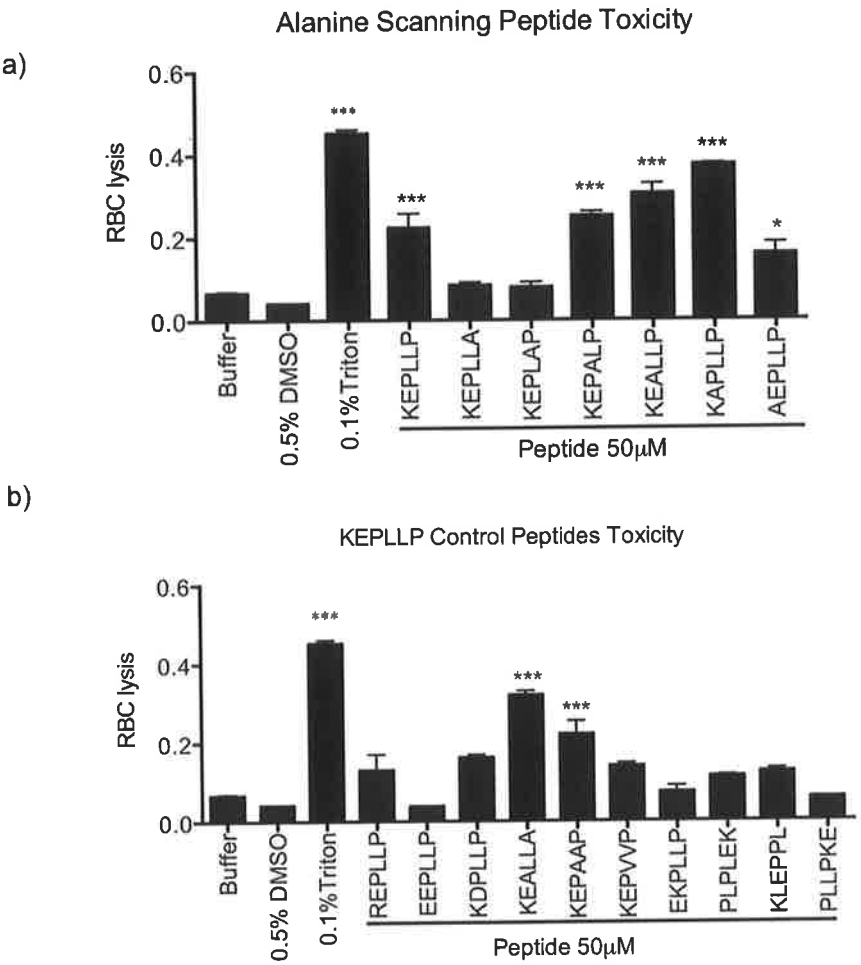


Figure 3.15 Effect of KEPLLP control peptides in RBC lysis assay. The effect of KEPLLP peptides on RBCs was measured at 50μM concentration. Alanine scanning peptides (a) and control peptides (b). Peptides were incubated with RBC for 15 minutes at 37°C, samples were centrifuged at 1000xg for 5 minutes. The supernatant was collected and analysed at 405nm absorbance for the presence of hemoglobin. Data represents mean ±SEM of N=3 individual expreiments, where *P<0.05, **P<0.001 and ***P<0.001 represent statistical significance values determined by One-way ANOVA. Significance was obtained by comparison of peptide or Triton treated RBC treatment with buffer treated RBC (negative control).

3.3 Discussion

In this chapter, SLIM peptides derived from JMD of E- and N-Cadherin have been designed and assessed to expand prior works regarding the role of cadherin in platelet function (Edwards et al., 2007). The data in this chapter demonstrate that cadherin-derived peptides were able to inhibit platelet function, but this bioactivity is not limited to parent peptide sequences.

Using palmitoylated peptides derived from important platelet proteins to target platelet function is a well-established method (Koloka et al., 2008, Aylward et al., 2006, Covic et al., 2002b, Edwards et al., 2007). Edwards *et al.* have used *in silico* methods to identify the potentially bioactive peptides from a large data set of platelet proteins (Edwards et al., 2007). For instance, one of the peptides derived from JMD of K-Cadherin exhibited significant anti-platelet activity. Therefore the activity of peptides derived from other cadherins on platelet function was explored using a similar approach. The effect of these peptides on platelets was analysed in platelet functional assays.

Initially, both platelet aggregation assays and a novel assay of platelet ATP secretion were utilized in this study. Although platelet aggregation represents a gold-standard assay of platelet function, the ATP secretion assay was demonstrated to be as good as platelet aggregation in detecting the effects of the peptides. Moreover, ATP secretion assay is a highly suitable assay for high throughput screening of peptides. In

experiments where both aggregation and secretion assays were used in parallel, the results were identical. However, because the secretion assay requires a lower assay volume, can deal with greater samples numbers in a shorter time frame and is easier to perform than aggregation. Therefore, the secretion assay was selected for all subsequent studies.

Based on the results from Edwards *et al.* it was suggested that cadherins might play a role in platelet activation (Edwards et al., 2007). Initial experiments presented in this chapter showed that peptides derived from the evolutionarily conserved JMD of E-and N-Cadherin also showed potent inhibition of TRAP induced platelet function. Some peptides are more potent and some are less potent. They show dose dependent inhibitory activity on both platelet ATP secretion and aggregation responses. In order to assess the specificity of these responses, a series of experiments were designed to assess sequence dependence, dose dependence and specificity of action of these peptides.

An ideal method to determine the sequence specific activity of any peptides is to design and assess the control peptides. In this chapter control peptides were designed using a method developed in computational biology department in University College Dublin (UCD). http.//bioware.ucd.ie/~cyclops/Fergal/tags/PepControls. Analysis of control peptides of E-Cad 1, 2 and N-Cad 2 led to several conclusions. E-Cad 1 and N-Cad 2 reverse charge peptides did not show any anti-platelet activity. This suggests positively charged residues are crucial for

activity but E-Cad 2 peptide did not obey this consensus. Both E-Cad 1 and N-Cad 2 peptides did not exhibit sequence specific activity because most of their control peptides also exerted an anti-platelet activity. Interestingly E-Cad 2 peptide did manifest sequence specific activity. Together, these results seemed to suggest that peptides could show non-specific effects on platelets.

In parallel, the truncation study identified that positive charges at the N-terminus are most likely to play a key role in modulating platelet function. In addition, this analysis also highlighted a short sequence KEPLLP, as a potent inhibitor of platelet function. This sequence had previously been identified as a dynamic binding site for P120-catenin in E-Cadherin JMD/P120-catenin interaction (Ishiyama et al., 2010). Interestingly, the LL motif within the KEPLLP peptide is involved in clathrin-mediated endocytosis of E-Cadherin (Miyashita and Ozawa, 2007b, Miyashita and Ozawa, 2007a). The binding of adaptor protein 2 (AP2) to LL motif leads to clathrin-mediated endocytosis of E-Cadherin (Kelly et al., 2008). AP-2 is a heterotetramer consisting of two large adaptins (α or β), a medium adaptin (μ), and a small adaptin (σ) (Kelly et al., 2008). The association of P120-catenin to E-Cadherin JMD will hide the LL motif from AP2 thereby preventing the internalization of E-Cadherin from the cell surface. Due to this key role of KEPLLP in E-Cadherin-P120-catenin interaction, the activity of KEPLLP was further assessed using control peptides to understand its effect on platelet function.

Analysis of the KEPLLP peptide series showed that replacement of the LL motif with two valines (KEPVVP) shows a similar effect to KEPLLP at 12.5μM. This response is expected because both leucine and valine are similar in hydrophobicity. In similar, alanine scanning peptide results also demonstrated that replacement of each leucine residue failed to inhibit platelet function. Taken togeteher, while these data could be seen to support role a for LL or hydrophobic motifs in the regulation of cadherin function, the observation that inbitory activity of random scrambled peptides (PLPLEK and KLEPPL) even at 12.5μM, make it difficult to draw any proper conclusion on specificity of KEPLLP peptide. However, this data suggests that there is a large component of non-specific activity associated with these peptides which could be a consequence of toxic properties associated with peptides.

In order to explore the potential toxicity of the peptides used in this study, a group of peptides was assessed in a standard assay of cellular toxicity, namely a LDH assay. Initial peptide toxicity analysis showed that cadherin-derived peptides were not toxic. However, the LDH assay require substantial damage to the platelets in order to detect the toxic effects. Peptides used in this study might cause subtle damage to the platelets which may not detect by LDH asssay. Therefore, the effect of these peptides was assessed in a more sensitive toxicity assay developed using RBC. The toxicity analysis of E-Cad 1,2 and N-Cad 2 and their control peptides using RBC (Table 3.2) identified that none of the peptides can induce damage to the RBC. This indicates the anti-

platelet activity of E-Cad 1,2 and N-Cad 2 peptides is not associated with toxicity. To further explore this, KEPLLP control peptides were also analysed in RBC assay to understand their toxicity effects. RBC lysis assay (but not LDH) of KEPLLP and its control peptides suggests some/many of the short peptides were able to induce the leakage of hemoglobin. This implies that peptides in the KEPLLP series are causing mild acute toxicity to the platelets which can not be detected by normal toxicity assays like LDH. Interestingly, none of the random scrambled peptides of KEPLLP caused damage to the RBC (PEPLEK and PLLPKE) but these peptides were potent inhibitors of platelet function. This is clearly suggests that activity is independent of toxicity and there may be multiple contributions to the inhibitory activity of cadherin-derived peptides.

The platelets response to cadherin-derived peptides is highly non-specific. The mechanism of action of these peptides is still unknown. Majority of cadherin-peptides used in this study are composed of several positive amino acids in their sequences. This is observation suggesting that cadherin-derived peptides may exhibit their effects on platelets similar to cationic anti-microbial peptides. Several cationic anti-microbial peptides mechanism of actions are well studied (Brogden, 2005). Anti-microbial peptides exert their effects on microorganisms by pore formation, inhibition of cell wall and nucleic acid synthesis (Brogden, 2005). In similar to cationic anti-microbial peptides, cadherin-derived peptides used in this study may cause pore formation on platelet surface

and inhibit their function. There are many studies that have demonstrated that a palmitoylated peptide approach was useful to understand the role of certain receptors like integrins and G-proteins coupled receptors (GPCR) in platelets (Koloka et al., 2008, Aylward et al., 2006, Covic et al., 2002b, Edwards et al., 2007, Stephens et al., 1998). Recent studies have shown that palmitoylated agonist peptides derived from PAR1 and C-X-C chemokine receptor type 4 (CXCR-4), required receptor expression for their activity (Covic et al., 2002a, Tchernychev et al., 2010). In this chapter, the study of cadherin-derived peptides and their effects on platelet function was started based on the findings of Edwards *et al.* (Edwards et al., 2007). Edwards *et al.* have previously reported a significant effect of a K-Cadherin-derived peptide on platelet function (Edwards et al., 2007). The presence of K-Cadherin in platelets had been suggested from platelet mRNA analysis (McRedmond et al., 2004). Interestingly, peptides designed based on the sequence of E- and N-Cadherin were potent inhibitors of platelet function similar to K-Cadherin peptides (Edwards et al., 2007). Therefore the nature of these effects was not explored in this chapter. However, to date, only one study has explored the presence of E-Cadherin in platelets. Elrod *et al.* demonstrated the presence of E-Cadherin in platelets (Elrod et al., 2003) and our initial E-Cadherin peptide data seemed to show a specific effect on platelet function. However, subsequent exploration regarding the sequence-dependence, motif-dependence or feature-dependence of the peptides, by exploring scrambled or alanine-scanned peptides, reverse-charged peptides or peptides in which non-charged AAs were scrambled,

indicated little relationship between the biological effects and the peptide sequence. This led us to question the specificity of the observed effects.

To our surprise, there are very few reports in the literature that address the sequence specific activity of parent peptides. Even the paper from our own laboratory had simply used a convenient control peptide for a study on the effects of the integrin-derived peptide Pal-KVGFFKR on platelet function. The control peptide used was Pal-KAAAAAR and it failed to affect platelet function in the assays reported (Stephens et al., 1998). In a subsequent study an alternative peptide was used comprising an irrelevant sequence from another platelet protein, Pal-EIIEDIKRHK, which differed in length from the experimental peptide (Bernard et al., 2009). Similarly, other studies with palmitoylated peptides used, single selected control peptides or no control peptide to support claims of biological activity (Martin et al., 2003, Covic et al., 2002a) Finally, the paper by Edwards *et al.* explored 43 peptides derived from platelet membrane proteins and designed a single control for some peptides (Edwards et al., 2007). The assessment of various truncated peptides in this chapter suggested that anti-platelet activity is probably associated with charged amino acids in the peptide sequence. Further analysis of this observation may have greater impact on our understanding of the non-specific inhibition of platelet function by palmitoylated peptides.

In future, in the design of bioactive peptides to target platelet function, investigators should consider the charged residue distribution within the

peptide sequence. In addition, analysis of possible control peptides of parent peptide at higher and lower concentrations needs to be considered.

In conclusion, although initially promising, the peptides assessed in this chapter seemed to exert largely non-specific effects on platelet function. Some of the non-specific effects may be explained by a toxic effect (observed as the ability of the peptides to cause lysis of red cells). However, this effect failed to explain all of the observed bioactivity. The role played by positively charged amino acids (AAs) in the peptides is significant. Even though the work in this chapter fails to explain the role of cadherins in platelet function, it demonstrates the previously unknown non-specific effects of palmitoylated peptides on platelet function.

Chapter 4

Identification and Characterization of VE-Cadherin in Human Platelets

4.1 Introduction

Platelet hemostasis is a tightly regulated process, which involves various platelet surface receptors. It has become apparent that platelets express a number of tight junction proteins (or cell adhesion molecules), which have been shown to play a critical role in platelet function. For example, platelets express Platelet Endothelial Cell Adhesion Molecule (PECAM) on their surface. PECAM has been shown to play role in platelet adhesion/aggregation at sites of minor endothelial damage in brain arterioles (Rosenblum et al., 1996).

In addition to PECAM, platelets have been shown to express another group of junction proteins on their surface, the cadherins. Cadherins are single-pass transmembrane cell adhesion molecules, which can form homophilic interactions. Based on sequence comparisons, classical cadherins are divided into two different sub types, type-I or type-II. Both type-I and type-II cadherins share homology in their homophilic interactions but differ at the molecular level (Patel et al., 2006).

Elrod *et al.* first demonstrated the presence of E-Cadherin on the platelet surface but did not study its role in platelet function (Elrod et al., 2003). More recently, Dunne *et al.* shown evidence for expression of K-Cadherin on the surface of platelets and identified a potential role in thrombus formation (Dunne et al., 2012). In addition, at the start of this study, we believed we were identifying specific effects of peptides derived from E- and N-Cadherin (Figure 3.6). However, as described in the previous

chapter 3, E- and N-Cadherins appeared to have profound effects on platelet function. To help explain these effects, it was necessary to characterize the expression of different cadherins on the platelet surface and investigate the effect of the identified cadherins on platelet function. The aim of this current study is to characterize the expression of different cadherins on the platelet surface and investigate the effect of the identified cadherins in platelet function.

4.2 Results

4.2.1 Identification of VE-Cadherins in human platelets

The analysis of washed platelet lysates using western blots identified that platelets could express VE- and K-Cadherins but not E- and N-Cadherins (Figure 4.1a). In these blots, a specific positive control was used for each cadherin. For E- and pan-Cadherin, MCF7 breast cancer cell lysate was used. For N-Cadherin, human kidney epithelial (HK-2) cell lysate was used. Human umbilical vein endothelial cells (HUVEC) lysate was used for VE-Cadherin and for K-Cadherin; a recombinant pure protein was used as a positive control. The western blot of K-Cadherin indicated that there was a reduced expression of K-Cadherin in activated platelet lysates compared to resting lysates (Figure 4.1a). However, densitometric analysis of K- and VE-Cadherin revealed that, there was no consistent reduction of expression of this protein in activated platelets (Figure 4.1b). A pan-cadherin antibody that can detect the multiple cadherins reacted with bands of a similar molecular weight to VE-and K-Cadherin (Figure 4.1). In addition VE-Cadherin expression on platelets also verified using

flow cytometry (Figure 4.1c). For flow cytometric analysis non-specific IgG antibody used as negative control and platelet integrin αIIbβ3 (SZ22) antibody used as positive control to demonstrate the sensitivity of flow cytometry (Figure 4.1c). As expected, there was a slight fluorescence shift in flow cytometric histogram compare to platelet integrin αIIbβ3. This is probably due to low copy number of VE-Cadherin in platelets (Figure 4.1c).

The actual molecular weight of VE-Cadherin is 140kD but the VE-Cadherin reactive bands from platelet lysates in western blot was observed at ~120kD. VE-Cadherin is a glycosylated protein (Geyer et al., 1999) so, the difference in the molecular weight might be due to the difference in glycosylation profile of VE-Cadherin in platelets. Overall, these results identified, for the first time, that platelets express VE-Cadherin, in addition to K-Cadherin.

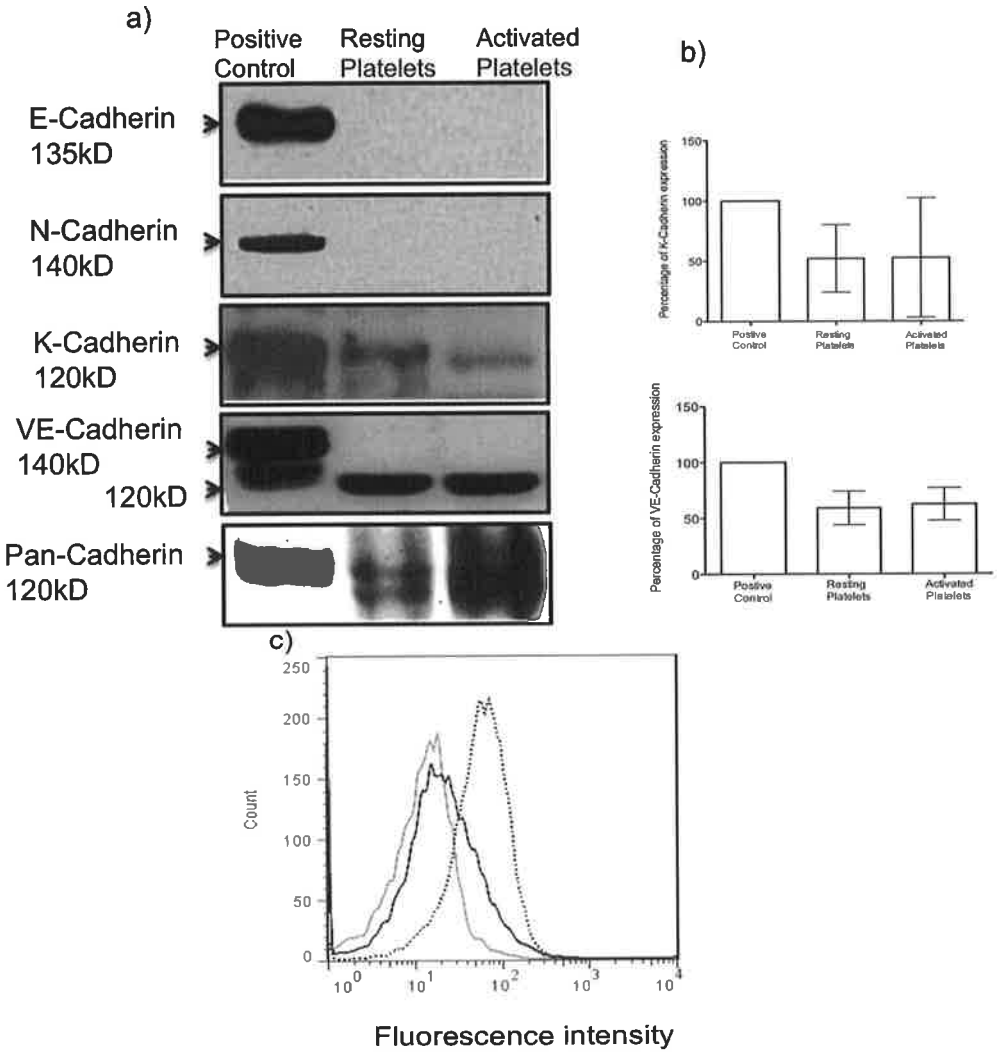


Figure 4.1 Identification of VE- and K-Cadherins in human platelets. Washed platelets were remained unactivated or were activated with TRAP (10μM) in platelet aggregometer for 5 minutes at 37°C. Following platelet activation lysates were prepared as described in methods and protein concentration was determined using Bio-Rad protein quantification assay. 20μg of platelet protein or other appropriate cell lysates were resolved using 7.5% SDS gels. a) Blots were incubated with antibodies against E, N, K, VE or pan-Cadherin as indicated. The positive-control cell lysates were MCF7 breast cancer cell lysate for E- and pan-Cadherin, HK-2 cell lysate for N-Cadherin and HUVEC lysate for VE-Cadherin. As a positive control for K-Cadherin, 1μg of a recombinant pure protein was used. The details of the antibodies are outlined in Table 2.2. b) Densitometry of K-Cadherin (up) and VE-Cadherin (bottom). For densitometric analysis of K-Cadherin data was normalized to positive control expression as 100%. Data represents platelet lysates from N=3 individual donors for E-, N- and Pan-Cadherins; N=6 for K- and VE-Cadherins. Error bars indicate mean ±SEM. c) Detection of VE-Cadherin using flow cytometry; washed platelets were incubated with non-specific mouse IgG (grey line), monoclonal VE-Cadherin antibody (black line) and integrin αIIbβ3 antibody (dotted black line) and analyzed in Flow cytometry as described in methods (section 2.2.20). Representative histogram of N=2 individual experiments was shown.

4.2.2 Characterization of VE-Cadherin antibody

Classical cadherins share 20 to 30% sequence homology in their amino acid (AA) composition. Due to the homology of the cadherin sequence, it is possible that the VE-Cadherin antibody might bind to a common epitope between cadherins. Therefore, the specificity of VE-Cadherin antibody was characterized using different cell lysates, which express other type of Cadherins. Western blots analysis of HK-2 (positive control for N-Cadherin), MDA MB-231 breast cancer cell (positive control for E-Cadherin), and HUVEC cell lysates (positive control for VE-Cadherin) identified that VE-Cadherin antibody can specifically binds to only VE-Cadherin but not to E- and N-Cadherins (Figure 4.2).

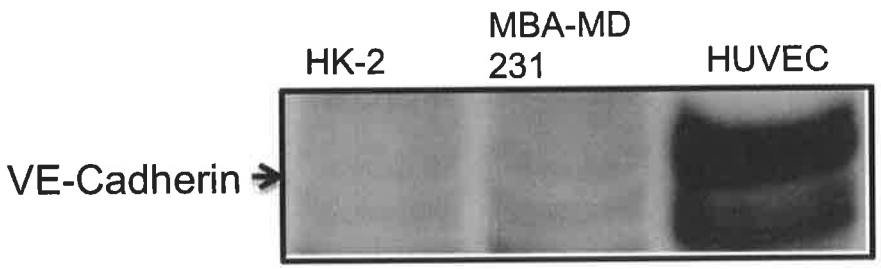


Figure 4.2 VE-Cadherin antibody characterization.
HK-2, MDA-MB 231 and HUVEC lysates were prepared as described in methods (section 2.2.6.1) and 20µg protein concentration of cell lysates was resolved using 7.5% gels. Blots were probed with VE-Cadherin antibody. A single blot shown is representative of N=3 individual experiments.

4.2.3 Confirmation of VE-Cadherin expression in human platelets

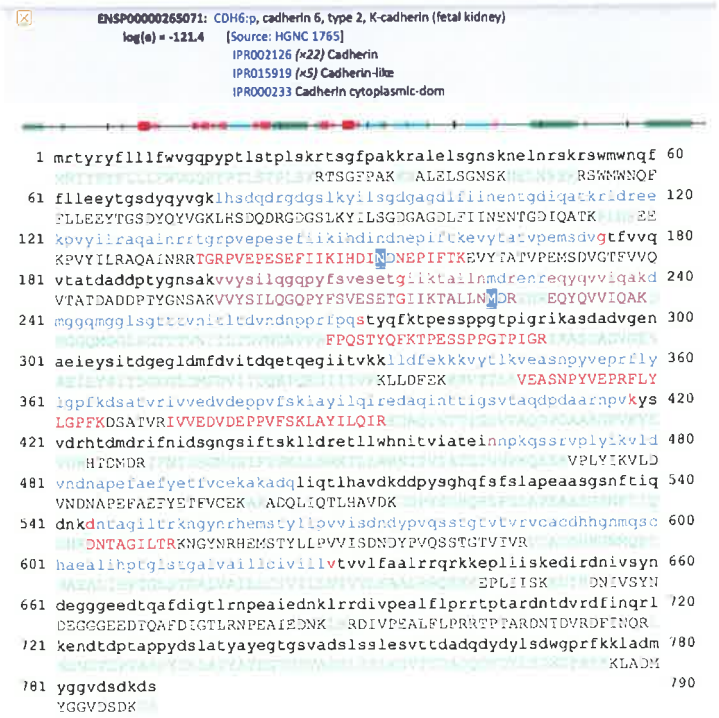
The expression of VE-Cadherin in platelets was further confirmed using mass spectrometric (MS) analysis of VE-Cadherin immunoprecipitates from platelet lysates. In these experiments, a recombinant K-Cadherin protein was analysed to demonstrate the ability and sensitivity of the MS

systems used. Figure 4.3 shows the profile of K-Cadherin protein and demonstrates the information available. In brief, peptides are identified in the MS outputs that, on comparison with the Uniprot sequence of the parent protein, can verify the presence of the protein. Peptides identified in MS were highlighted red in Uniprot sequence. Next, VE-Cadherin was immunoprecipitated from platelet lysates using Protein-A agarose beads coupled to the specific anti-human VE-Cadherin antibody.

Immunoprecipitated samples were resolved using SDS-PAGE and bands at the expected molecular weight region were excised and digested with trypsin as described in methods (section 2.2.7) (Huynh et al., 2009). Resulting peptides were analysed using an Orbitrap XL (Thermofisher) mass spectrometer for the presence of VE-Cadherin or other cadherin-derived peptides. Figure 4.5 shows the profile of the peptides identified from platelet VE-Cadherin immunoprecipitates. In addition, immunoprecipitates of K-Cadherin from platelet lysates using anti-human K-Cadherin antibody were assessed in similar manner (Figure 4.4). The MS analysis of cadherin immunoprecipitates was carried out in collaboration with Prof. Achim Treumann from Newcastle University. MS analysis confirmed the expression of VE- and K-Cadherin in human platelets. The MS analysis of immunoprecipitates from platelet lysates identified VE-Cadherin peptides with log(e) value of -69.4, rI value of 10 (Figure 4.5) and for K-Cadherin log(e) and rI values were -18.4 and 3 respectively (Figure 4.4). The log(e) value indicates the confidence of peptide identification and rI value represents the number of peptides

identified. These values obtained in MS analysis for VE- and K-Cadherin indicated that VE-Cadherin is more abundant compared to K-Cadherin in platelets. In addition, recombinant K-Cadherin analysis in MS was identified with higher log(e) value -121.1 and rl value of 16 (Figure 4.3).

a)



b)

Identified Peptides

spectrum	log(e)	log(i)	m+h	delta	ζ	sequence	n
456.1	-5.3	4.01	1601.8533	-0.0008	2/3	lnrr[135] TGRPVEPESE FIIK [148]ihdi	(60)
455.1	-3.8	4.21	1601.8533	-0.0019	3/3	lnrr[135] TGRPVEPESE FIIK [148]ihdi	(60)
540.1	-8.3	3.84	1287.6831	0.0020	2/2	rtgr[118] PVEPESEFII K [148]ihdi	(1)
443.1	-4.1	3.29	1556.7591	-0.0021	2/3	fiik[149] IHDNDNEPI FTK [161]evyt	(115)
417.1	-2.1	3.90	1555.7751	-0.0015	3/3	fiik[149] IHDINDNEPI FTK [161]evyt	(115)
629.1	-2.8	3.36	1266.6729	0.0012	2/2	nsak[197] VVYSILQGQP Y [207]fsve	(0)
504.1	-3.5	3.75	1209.6361	-0.0008	2/2	gqpy[208] FSVESETGII K [218]tail	(0)
197.1	-2.2	4.61	949.4771	0.0003	2/2	gsik[219] TALLNMDR [226]enre	(14)
197.2	-2.2	4.61	949.4812	-0.0038	2/2	gsik[219] TALLNMDR [226]enre	(14)
372.1	-3.2	3.69	1205.6525	-0.0006	2/2	renr[230] EQYQVVIQAK [239]dmgg	(152)
416.1	-2.8	4.56	1145.5626	-0.0003	2/2	nppr[268] FPQSTYQFK [276]tpes	(11)
240.1	-4.0	3.63	1295.6590	0.0010	2/2	yqft[277] TPESSPPGTP IGR [289]kas	(248)
251.1	-4.3	3.48	1260.6219	0.0002	2/2	ytlk[347] VEASNPYVEP R [357]fyl	(195)
624.1	-1.6	3.99	984.5553	-0.0004	2/2	vepr[358] FLYLGPFK [365]dsal	(96)
597.1	-8.7	3.44	1572.8156	0.0002	2/2	atvr[372] IVVEDVDEPP VFSK [385]layi	(189)
594.1	-2.2	3.55	989.6142	-0.0002	2/2	vlsk[386] LAYILQIR [393]edaq	(63)
292.1	-1.7	4.51	960.5109	0.0006	2/2	qdnk[544] ONTAGILTR [552]kngy	(10)

c)

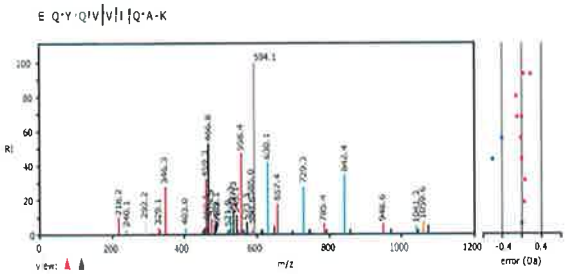


Figure 4.3 Orbitrap Mass spectrometric analysis of recombinant K-Cadherin. Recombinant K-Cadherin protein was resolved using SDS-PAGE. A 0.5 cm band centred on the expected molecular weight (120kDa) was excised and digested with trypsin. The resulting peptides were analysed using an Orbitrap XL (Thermofisher) at a resolution of 30,000. Peptides identified were compared to the human genome using the global proteome machine as described in methods (section 2.2.7.8). a) The sequences of K-Cadherin peptides that were identified in MS are highlighted in red in the Uniprot sequence. The intensity of the red is related to the confidence score –log(e). b) A list of peptide sequences identified in mass spectra for K-Cadherin is shown. Only peptides with a confidence value (-log(e)) of <-3.0 were considered in our analysis. The amino acids (AAs) illustrated in blue text correspond to the sequences identified by MS analysis. Bold text suggests a modified AA was observed. The 'n' value refers to the number of observation of each peptide during the experimental run. c) The profile of a single sample-peptide identified is shown. The error on estimates is less than 0.4 Daltons on each peptide fragment identified.

a)

b)

Identified Peptides

spectrum	log(e)	log(I)	m+h	delta	ζ	sequence	n
959.1	-2.8	3.64	1295.6590	-0.0050	2/2	yqfk^{277} TPESSPPGTP IGR ^{189}kas	(248)
988.1	-1.1	3.71	1260.6219	0.0045	2/2	ytlk^{347} VEASNPYVEP R ^{357}flyl	(195)
2401.1	-5.3	3.67	1572.8156	-0.0044	2/2	atvr^{372} IVVEDVDEPP VFSK ^{385}layl	(189)

c)

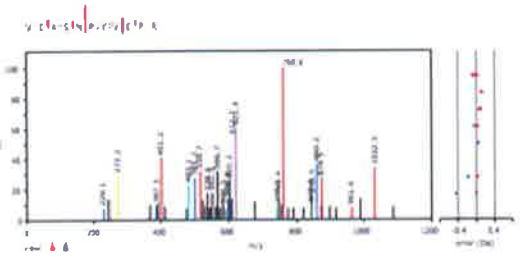


Figure 4.4 Orbitrap Mass spectrometric analysis of K-Cadherin immunoprecipitated from platelet lysate. K-Cadherin protein was immunoprecipitated from platelet lysates using a monoclonal antibody against K-Cadherin and samples were resolved using SDS-PAGE. A 0.5 cm band centred on the expected molecular weight (120kDa) was excised and digested with trypsin. The resulting peptides were analysed using an Orbitrap Orbitrap XL (Thermofisher) at a resolution of 30,000. Peptides identified were compared to the human genome using the global proteome machine as described in methods (section 2.2.7.8). a) The sequences of K-Cadherin peptides that were identified in MS are highlighted in red in the Uniprot sequence. The intensity of the red is related to the confidence score –log (e). b) A list of peptide sequences identified in mass spectra for K-Cadherin is shown. Only peptides with a confidence value (-log (e)) of <-3.0 were considered in our analysis. The AAs illustrated in blue text correspond to the sequences identified by MS analysis. Bold text suggests a modified AA was observed. The 'n' value refers to the number of observation of each peptide during the experimental run. c) The profile of a single sample peptide identified is shown. The error on estimates is less than 0.4 Daltons on each peptide fragment identified.

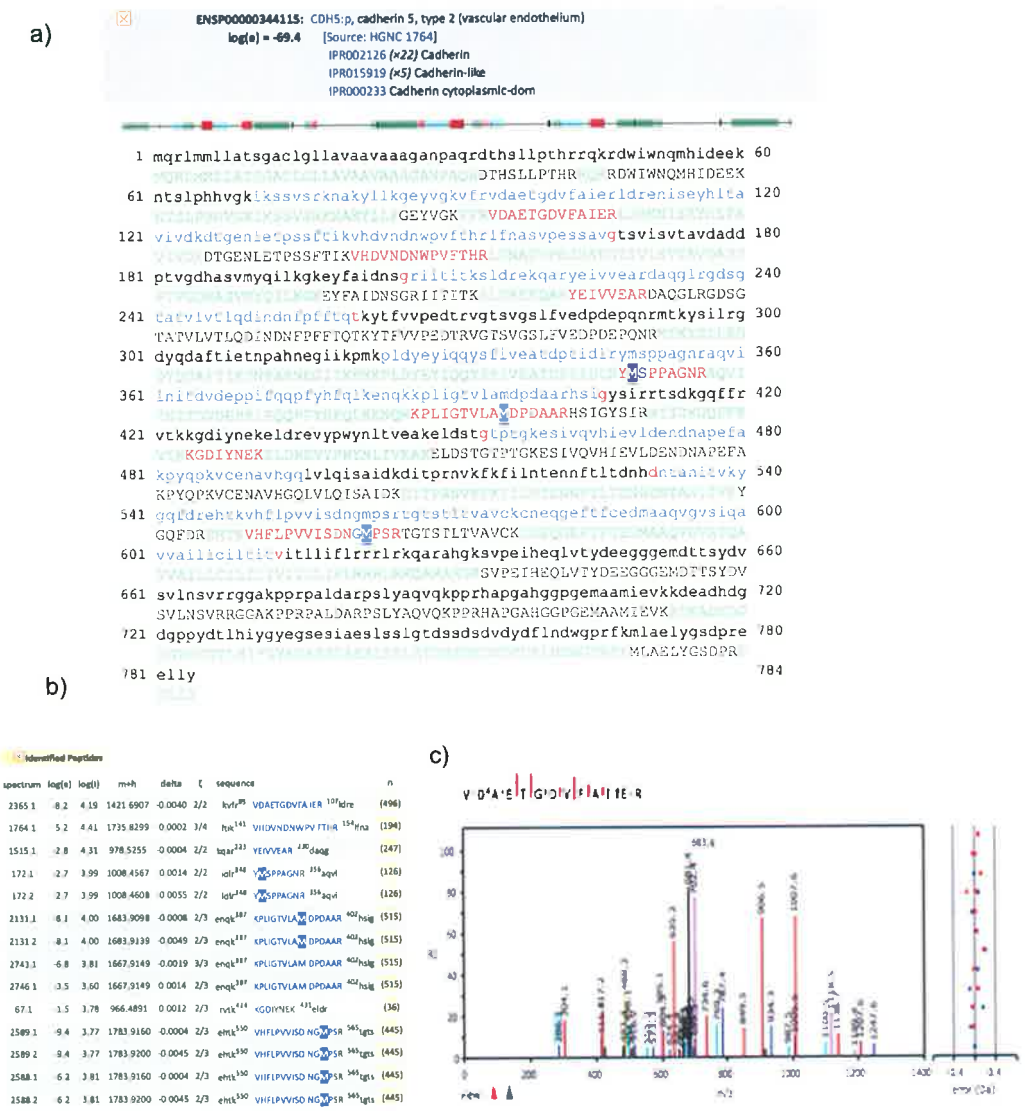


Figure 4.5 Orbitrap Mass spectrometric analysis of VE-Cadherin immunoprecipitate from platelet lysate. VE-Cadherin protein was immunoprecipitated from platelet lysates using monoclonal anti-human VE-Cadherin antibody and samples were resolved using SDS-PAGE. A 0.5 cm band centred on the expected molecular weight (120kDa) was excised and digested with trypsin. The resulting peptides were analysed using an Orbitrap XL (Thermofisher) at a resolution of 30,000. Peptides identified were compared to the human genome using the global proteome machine as described in methods (section 2.2.7.8). a) The sequences of VE-Cadherin peptides that were identified in MS are highlighted red in the Uniprot sequence. The intensity of the red is related to the confidence score –log (e). b) A list of peptide sequences identified in mass spectra for VE-Cadherin is shown. Only peptides with a confidence value (-log (e)) of <-3.0 were considered in our analysis. The AAs illustrated in blue text correspond to the sequences identified by MS analysis. Bold text suggests a modified AA was observed. The 'n' value refers to the number of observation of each peptide during the experimental run. c) The profile of a single sample-peptide identified is shown. The error on estimates is less than 0.4 Daltons on each peptide fragment identified.

4.2.4 Identification of cadherin-associated protein P120-catenin in human platelets

Previous results confirmed the expression of VE-Cadherin in human platelets. In endothelial cell systems, the interaction between VE-Cadherin and P120-catenin is necessary for endothelial barrier function (Iyer et al., 2004). It is well known that most classical cadherins bind to P120-catenin and β-catenin or junctional plakoglobin via their cytoplasmic tails (Harris and Tepass, 2010). β-catenin then binds to α-catenin, which connects the cadherins to the actin cytoskeleton to maintain the stability of cadherins at cell surface. β-catenin has previously been identified in platelets and it plays a role in the Wnt signaling pathway (Steele et al., 2009). Therefore in this study, platelet lysates were further analysed for the presence of cadherin-associated proteins P120-catenin, α-catenin and junctional plakoglobin. The analysis of platelet lysates using western blots identified P120-catenin but not α-catenin and junctional plakoglobin (Figure 4.6a) in human platelets. In addition platelet lysates were also analysed for β-catenin (N=1) (Figure 4.6b).

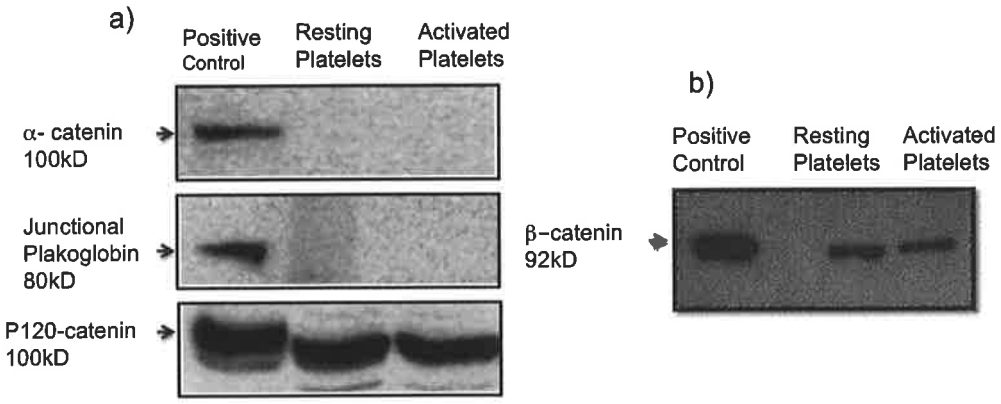


Figure 4.6 Identification P120-catenin in human platelets. Washed platelets were remained unactivated or were activated with TRAP (10μM) in platelet aggregometer for 5 minutes at 37°C. Following platelet activation lysates were prepared as described in methods (section 2.2.6.1) and protein concentration was determined using Bio-Rad protein quantification assay. 20μg of platelet protein concentration or other appropriate cell lysates were resolved using 7.5% SDS gels. HUVEC cell lysates for α-catenin, junctional plakoglobin, MBA-MD 231 cell lysates for P120 and β-catenin were used as positive controls. Proteins were electroblotted onto a PDVF nylon membrane. Blots were subsequently incubated with different antibodies against α-catenin, junctional plakoglobin and P120- catenin (a) and β-catenin (b). Details of the antibodies listed in (Table 2.2). Blots are representative of N=3 independent experiments for α-catenin, junctional plakoglobin, P120-catenin and N=1 for β-catenin.

4.2.5 Identification of VE-Cadherin role in platelet activation

The function of VE-Cadherin on endothelial cells is altered upon exposure to inflammatory mediators or activators, such as thrombin and induce the vascular permeability (Rabiet et al., 1996). VEGF induce the phosphorylation of VE-Cadherin followed by receptor down regulation and allow the cell sprouting and cell migration during angiogenesis (Esser et al., 1998). Therefore, the expression of VE-Cadherin was examined in response to platelet activation induced by various agonists in order to identify if the cadherin expression was affected by platelet activation. In addition, the phosphorylation of VE-Cadherin was also verified upon platelet activation using 4G10 phosphorylation detection antibody. Platelets were activated with three different agonists TRAP (10μM) for increasing amounts of time (0 to 45

minutes), collagen (190µg/ml) (15 minutes) and U46619 (2.5µM) (thromboxane mimetic) (15 minutes) and VE-Cadherin expression was quantified using western blots (Figure 4.7a). β-actin antibody was used to determine the equal loading in the gel (Figure 4.7a). Densitometric analysis of western blots identified that there is slight down regulation VE-Cadherin expression upon platelet activation compared to resting samples (Figure 4.7b). However, there was no significance was observed between activated samples and unactivated samples. In addition, VE-Cadherin did not become phosphorylated upon platelet activation (Figure 4.7c). These results suggest that VE-Cadherin is not involved in platelet activation in response to standard platelet agonists. However, it is possible that VE-Cadherin plays a role in platelet activation by non-standard agonists such as cancer cells or bacteria (Cox et al., 2011). It was not within the scope of this study to explore all possible platelet agonists, but it will be of interest to observe if a role for VE-Cadherin is identified in platelet activation in the future.

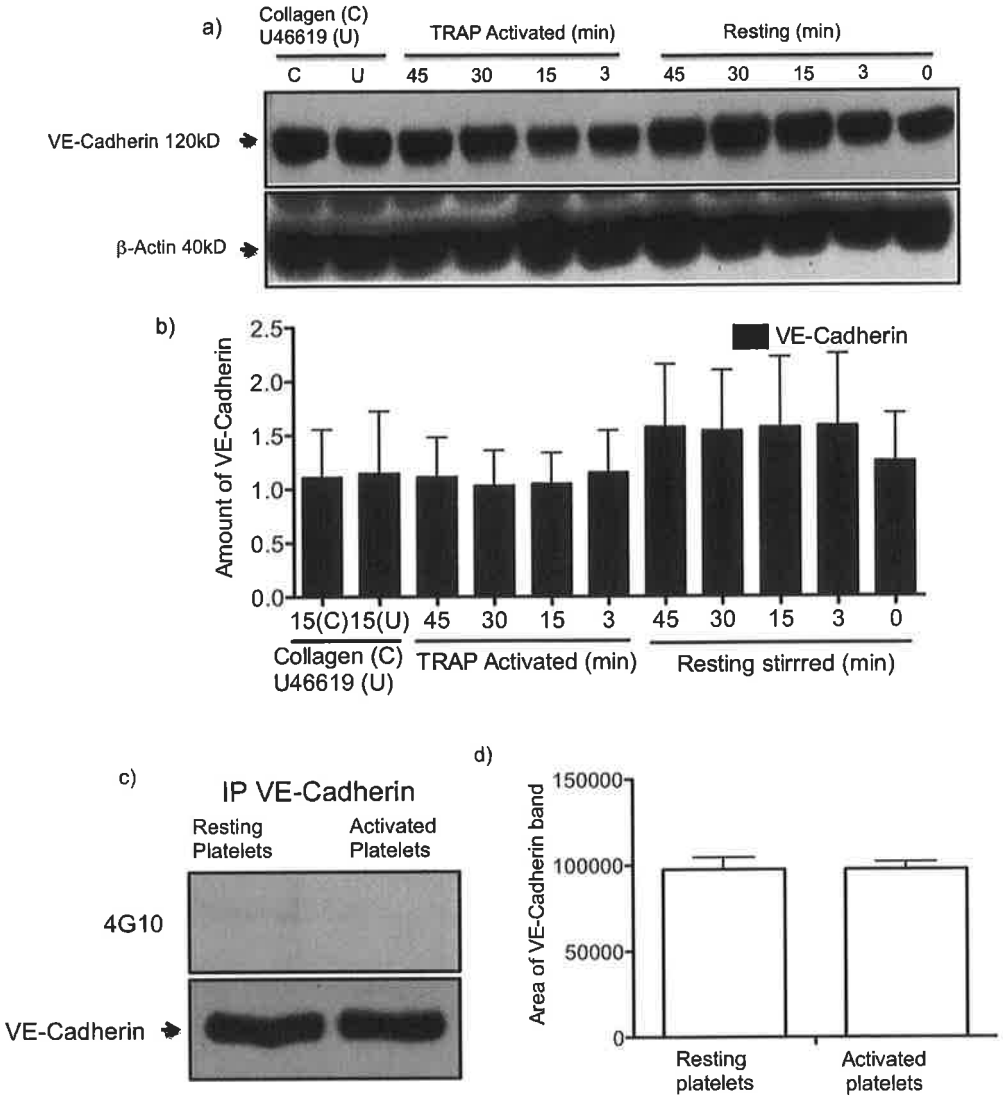


Figure 4.7 VE-Cadherin is not altered upon platelet activation. Washed platelets were untreated or were activated with different agonists (TRAP, collagen or U46619) at the indicated time points using the platelet aggregation technique as described in methods (section 2.2.2). Unactivated platelets were also stirred in an aggregometer (resting stirred) similar to activated platelets, but without any agonist at the indicated time points. Platelet lysates was prepared after aggregation and protein concentration was determined using Bio-Rad assay. 20μg of protein of this lysate was resolved using 7.5% SDS gels and proteins were electroblotted onto a PDVF nylon membrane. Blots were subsequently incubated with the indicated antibodies. a) Expression of VE-Cadherin at different stages of platelet activation (upper panel) and equal protein loading quantified by reprobing of same blot with β-actin (lower panel). b) Densitometric analysis of VE-Cadherin expression in various platelet samples was calculated described in methods (section 2.2.8). Data represents results from mean ±SEM of N=5 individual donors. c) For VE-Cadherin phosphorylation, platelets were activated with TRAP (10μM) for 15 minutes and platelet lysates were prepared as described in methods (2.2.6.1). VE-Cadherin was immunoprecipitated with an antibody from resting and TRAP activated platelet lysates as described in methods (section 2.2.6.3). Samples were resolved using 7.5% SDS-PAGE, blots were incubated with phosphotyrosine antibody (4G10) (up) and VE-Cadherin expression was cross checked with reprobing of same blot with VE-Cadherin antibody (bottom). Blots are representative of N=3 individual experiments and area of VE-Cadherin bands in resting and activated platelets was calculated using image J (d).

4.2.6 Effect of VE-Cadherin blocking antibody on platelet aggregation.

Platelet aggregation is an ultimate final step of platelet activation. Platelet aggregation is mediated by binding of adjacent platelets via fibrinogen bridges between integrin αIIbβ3 molecules (Jackson, 2007) and it can be blocked by the integrin inhibitor, Abciximab. VE-Cadherin is known to form homophilic interactions with neighbouring cells (Taveau et al., 2008). Dunne *et al.* have suggested that blocking of K-Cadherin can inhibit the platelet aggregation (Dunne et al., 2012). Therefore, a novel role of VE-Cadherin to facilitate platelet interaction in platelet aggregation was suggested. This was investigated using a specific blocking antibody, BV9 (Corada et al., 2001). However, blocking of VE-Cadherin did not influence platelet aggregation induced by TRAP (2μM) (Figure 4.8 green bars). In contrast, the integrin inhibitor, Abciximab (2.5μg/ml) reduced TRAP-induced platelet aggregation to 40% (Figure 4.8 red bars). Moreover, there was no synergic response observed in the presence of a combination of low dose of Abciximab (2.5μg/ml) and the VE-Cadherin antibody BV9 (40μg/ml) (Figure 4.8 green bars) compared to a negative control (non-specific mouse antibody; Figure 4.8 blue bars). Thus, it appears that VE-Cadherin is not involved in platelet aggregation.

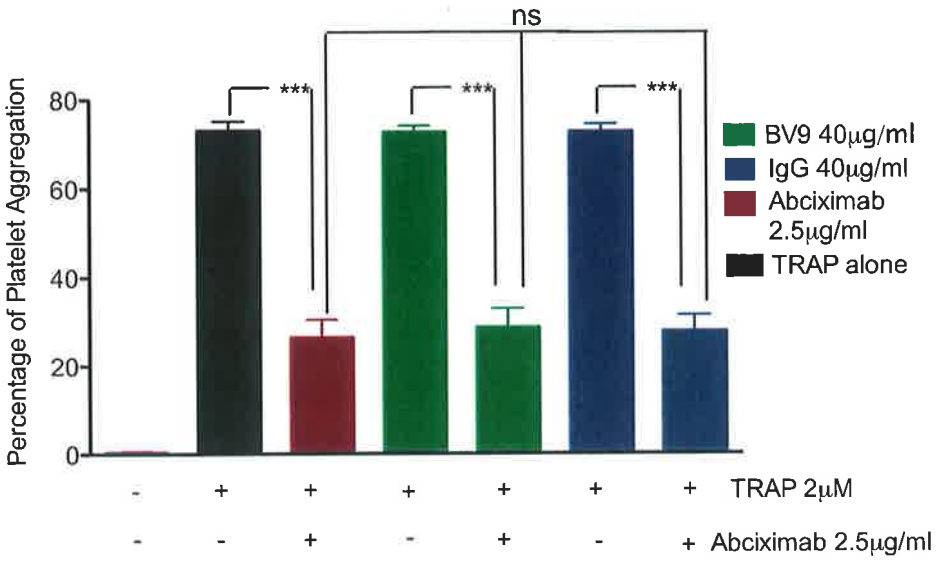


Figure 4.8 VE-Cadherin blocking antibody did not inhibit platelet aggregation. Washed platelets were treated with Abciximab (2.5µg/ml) (red bars) or BV9 (40µg/ml) (green bars) or non-specific mouse IgG (40µg/ml) (blue bars) for 15 minutes before activation with TRAP (2µM). Platelets were also treated in combination with Abciximab+BV9 and Abciximab+non-specific mouse IgG for 15 minutes at 37°C. Following incubation with several antibodies, platelets were activated with TRAP (2µM) and aggregation was recorded. Data is presented as mean ±SEM of N=3 individual experiments, where ***P<0.0001 is an indicator of significance calculated using One-way ANOVA.

4.2.7 Characterization of platelet adhesion to immobilized VE-Cadherin

To determine whether platelets can adhere to immobilized VE-Cadherin, washed platelets were allowed to adhere to immobilized proteins. Amount of platelet adherence was measured using phosphatase assay. Adherence was normalized to expressing platelets adhesion to fibrinogen as 100%. Platelets adhered to immobilized VE-Cadherin was similar to fibrinogen (Figure 4.10a) and adhesion to 1 and 2 µg/ml VE-Cadherin was also similar (Figure 4.10a). Therefore 1µg/ml VE-Cadherin was chosen for further analysis. To investigate the binding to immobilized VE-Cadherin, platelets were pre-treated with blocking antibody against VE-Cadherin BV9 (40µg/ml) and non-specific mouse IgG (40µg/ml) for 30 minutes before being allowed to adhere to immobilised VE-Cadherin for 45 minutes. Adherence was normalized to

amount of platelet adhesion to VE-Cadherin as 100% in the absence of any antibody. The antibody against VE-Cadherin (BV9) failed to inhibit the platelet adhesion to immobilized VE-Cadherin (Figure 4.10c). Platelets bind to proteins such as fibrinogen via platelet integrin αIIbβ3 in a RGD sequence dependent manner. In here, I observed RGD motif (Figure 4.9) in VE-Cadherin second extracellular domain. To investigate whether this RGD motif plays a role in platelet adhesion to VE-Cadherin, washed platelets were pre-treated with a synthetic RGD peptide and allowed to adhere to immobilized VE-Cadherin as above. The RGD (150μg/ml) peptide significantly inhibited platelet adhesion to immobilised VE-Cadherin (Figure 4.10c). In addition the role of platelet integrin αIIbβ3 in adhesion of platelets to VE-Cadherin was investigated using the known integrin blocking antibody αIIbβ3, Abciximab. Pre-treatment of platelets with Abciximab (5 and 20μg/ml) also significantly inhibited the platelet adhesion to VE-Cadherin (Figure 4.10c). Together, these results suggest that VE-Cadherin is not binding in a homophilic manner in this assay. Instead, VE-Cadherin is binding to platelets via platelet integrin αIIbβ3 in RGD dependent manner. In these assays, adhesion of platelets to 20μg/ml fibrinogen was also assessed as positive control (Figure 4.10b). Platelet adhesion to fibrinogen was significantly inhibited by Abciximab (20μg/ml), RGD (150μg/ml) but not with BV9 (40μg/ml) and IgG (40μg/ml) (Figure 4.10b). The failure of BV9 to inhibit platelet adhesion to VE-Cadherin is interesting. Multiple domains from extracellular regions mediate homophilic binding of VE-Cadherin (Corada et al., 2001). BV9 binds to the epitope in between 1-268 AA located in extracellular domain (EC) 1 to 3 of VE-Cadherin (Figure 4.9) and impairs its function in endothelial cells (Caveda et al., 1996). Corada *et al.*

suggested that BV9 binds to EC3 and 4 of VE-Cadherin (Corada et al., 2001). However, the accurate binding domain of BV9 is still unclear (Figure 4.9). Its failure to inhibit platelet adhesion to immobilized VE-Cadherin may indicate that the BV9 binding site does not overlap with the RGD motif.

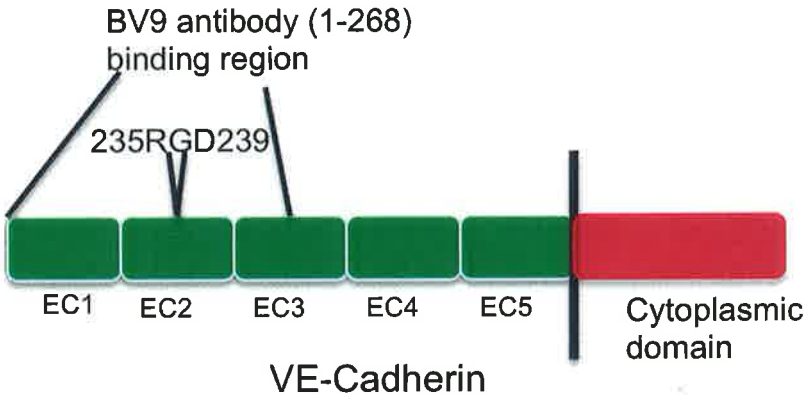


Figure 4.9 Graphical representation of VE-Cadherin extracellular domain (EC1 to 5) and cytoplasmic domain. RGD motif in EC2 and blocking antibody of VE-Cadherin (BV9) binding site in EC1 -3 are shown.

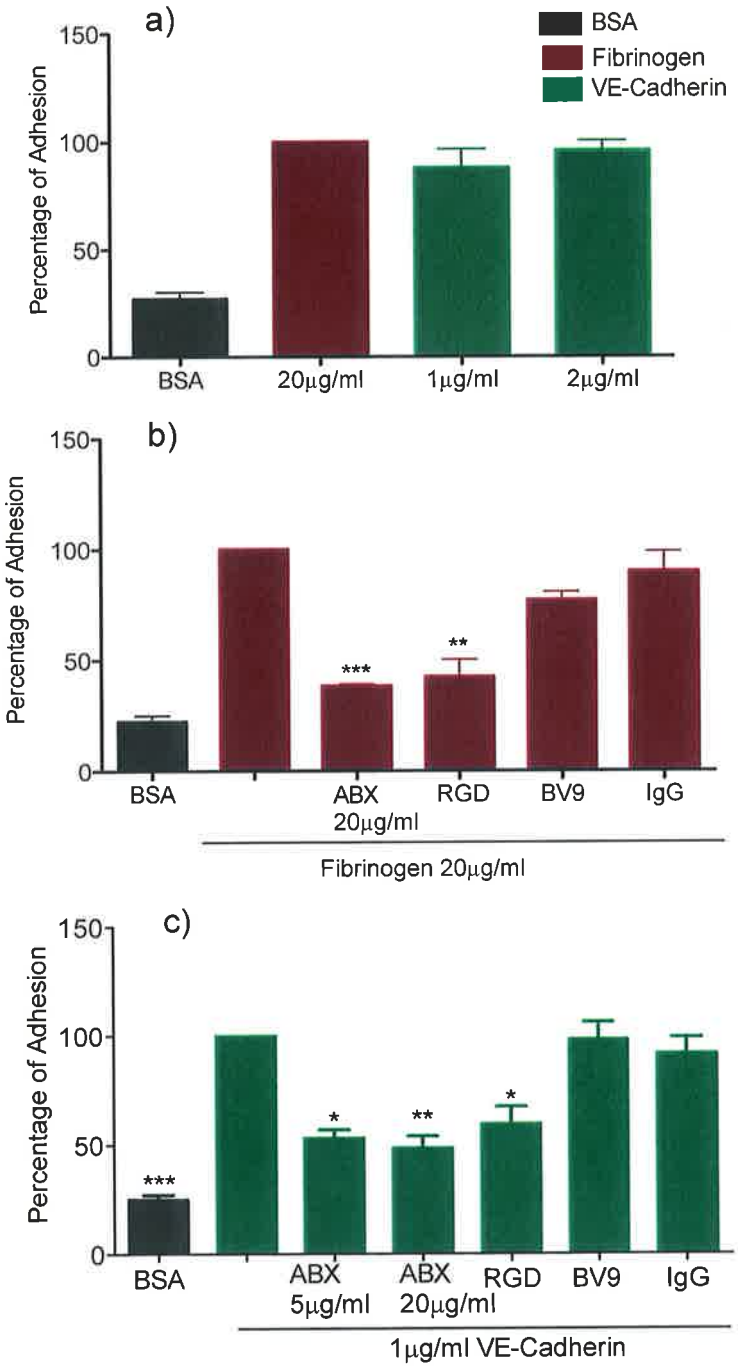


Figure 4.10 Platelets binds to VE-Cadherin is αIIbβ3 dependent. Clear 96-well plates were coated with VE-Cadherin (1 and 2µg/ml), fibrinogen (20µg/ml) and BSA (3%) overnight at 4°C. After coating, wells were blocked with 1% BSA for 1 hour at 37°C. Washed platelets were pre-treated with Abciximab (5 or 20µg/ml), RGD (150µg/ml), BV9 (40µg/ml) and non-specific mouse IgG (40µg/ml) for 30 minutes. Treated and non-treated platelets were allowed to adhere to immobilized proteins for 45 minutes at 37°C. The amount of platelet adhesion was measured using acid the phosphatase assay as described in the methods (section 2.2.15). a) Adhesion of platelets to BSA, fibrinogen and VE-Cadherin. b) Adhesion of platelets to BSA fibrinogen. c) Adhesion of platelets to BSA and VE-Cadherin. Data shown is mean ±SEM of N=6 individual experiments, where *P<0.05, **P<0.001 and ***P<0.0001 indicators of significance calculated using One-way ANOVA. Significance was obtained by comparison of platelet adhesion to immobilized fibrinogen or VE-Cadherin in the absence of any antibody or RGD peptide.

4.2.8 Determination of the role of αIIbβ3 in binding to VE-Cadherin

The role of αIIbβ3 in platelet adhesion to VE-Cadherin was further characterized using Chinese hamster ovary (CHO) cells stably transfected with wild-type integrin αIIbβ3 (CHO-FF), or a constitutively activated integrin αIIbβ3 variant (CHO-AA) or mock-transfected cells (CHO-MOCK), which do not express αIIbβ3 (Aylward et al., 2006) (Figure 4.11a). To examine the role of αIIbβ3, clear 96-well plates were coated with 1µg/ml VE-Cadherin and CHO cells were allowed to adhere in the presence and absence of RGD or Abciximab. The binding of integrin-expressing cells, compared to CHO-MOCK cells was assessed. Both CHO AA and FF cells were able to adhere to VE-Cadherin compared to BSA but their adhesion was significantly reduced by Abciximab (20µg/ml) and RGD (150µg/ml) (Figure 4.11b). In addition CHO mock cells did not show any adhesion to VE-Cadherin (Figure 4.11b). These results identified that platelet integrin αIIbβ3 can interact with VE-Cadherin via an RGD dependent mechanism. However, they appeared to be no difference between the ability of CHO-AA and CHO-FF cells to bind. These results demonstrate that binding is independent of the activation state of the integrin.

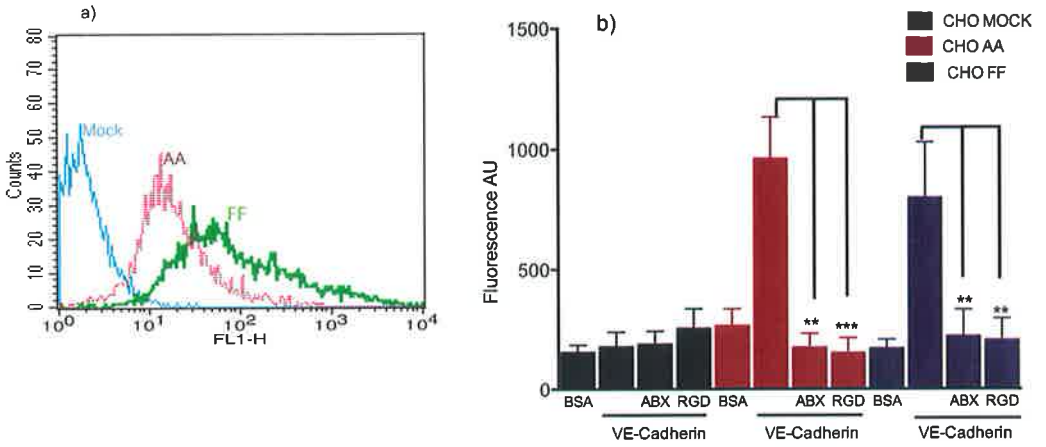


Figure 4.11 Platelet integrin αIIbβ3 expressing CHO cells adhesion to immobilized VE-Cadherin. a) Analysis of the expression of platelet integrin αIIbβ3 on the surface of CHO cells using flow cytometry. CHO-FF (resting integrin; green trace), CHO-AA (activated integrin; pink trace) and Mock (No integrin; blue trace). CHO cells were treated with FITC label conjugated αIIbβ3 (CD41a) antibody for 10 minutes and the expression of integrin was measured using flow cytometry as described in methods (section 2.2.18) and amount integrin expression expressed as fluorescence intensity (FL1-H). Flow cytometry histogram is a representative of N=2 individual experiments. b) Adhesion of CHO cells to immobilized 1µg/ml VE-Cadherin in the presence and absence of Abciximab (20µg/ml) and RGD (150µg/ml). Adhesion assay was performed as described in methods (2.2.19). Error bars represents mean ±SEM of N=5 individual experiments. **P<0.001 and ***P<0.0001 indicate the significance calculated using One-way ANOVA.

4.2.9 Analysis of platelet morphology on recombinant VE-Cadherin

To further understand the platelet binding to VE-Cadherin, the ability of platelets to spread on immobilized VE-Cadherin was observed. Platelets were able to adhere and spread on VE-Cadherin in a manner very similar to that observed for spreading on fibrinogen (Figure 4.12). This interaction between platelets-VE-Cadherin might play role in endothelial cell adhesion to platelets during hemostasis.

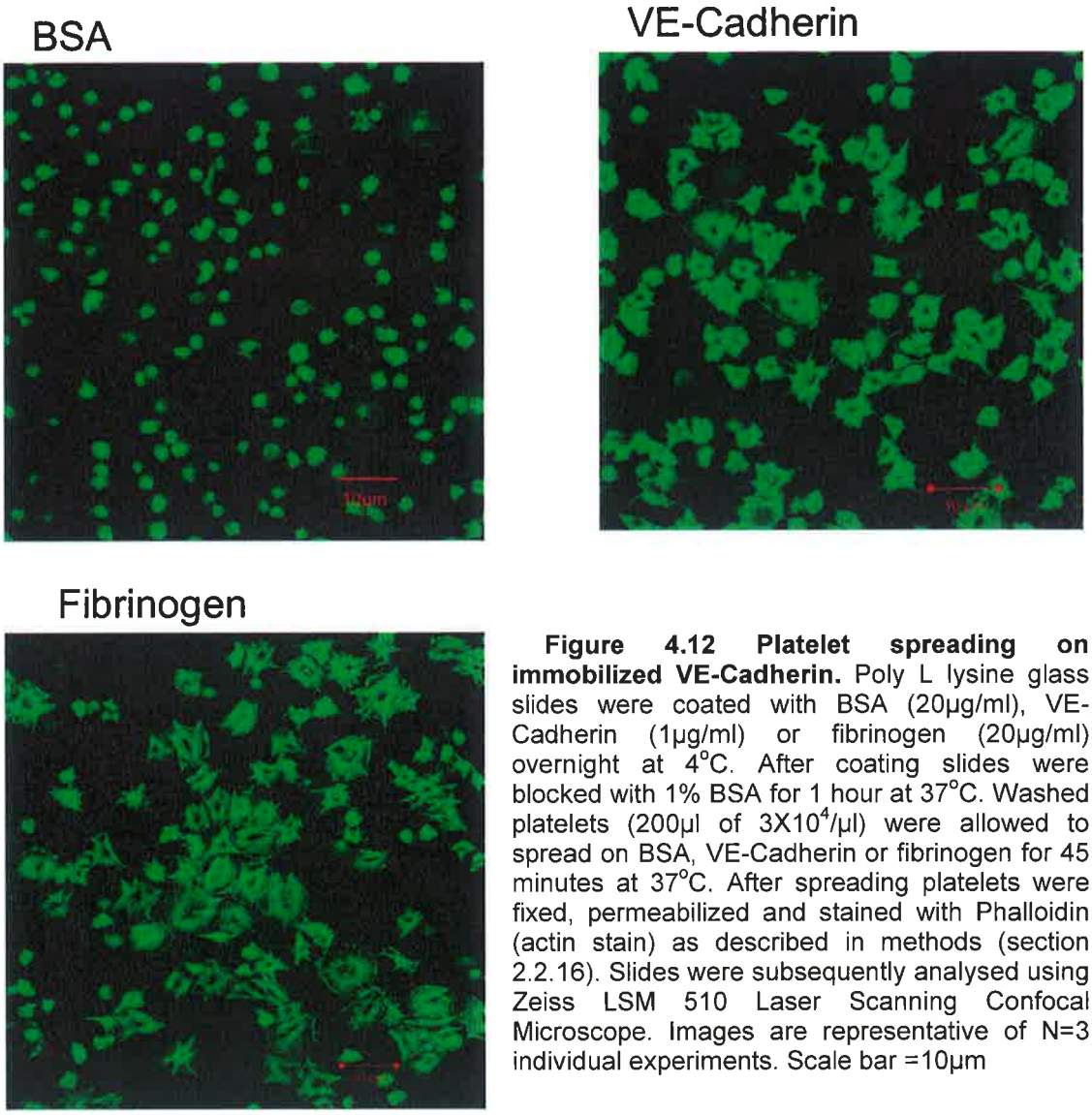


Figure 4.12 Platelet spreading on immobilized VE-Cadherin. Poly L lysine glass slides were coated with BSA (20µg/ml), VE-Cadherin (1µg/ml) or fibrinogen (20µg/ml) overnight at 4°C. After coating slides were blocked with 1% BSA for 1 hour at 37°C. Washed platelets (200µl of $3X10^4$/µl) were allowed to spread on BSA, VE-Cadherin or fibrinogen for 45 minutes at 37°C. After spreading platelets were fixed, permeabilized and stained with Phalloidin (actin stain) as described in methods (section 2.2.16). Slides were subsequently analysed using Zeiss LSM 510 Laser Scanning Confocal Microscope. Images are representative of N=3 individual experiments. Scale bar =10µm

4.2.10 Assessment of role of VE-Cadherin in activated platelets binding endothelial cells

Platelet reactivity is commonly regulated by the endothelium of blood vessels. Endothelial cells secrete nitric oxide to inhibit platelet activation in the vicinity of intact (undamaged) endothelial cells. However, under certain conditions activated platelets can bind to intact endothelial cells; this interaction is primarily mediated by fibrinogen (Bombeli et al., 1998, Mine et al., 2001).

Activation of endothelial cells with growth factors such as VEGF promotes the adhesion and activation of platelets (Verheul et al., 2000). To investigate a potential role for the VE-Cadherin in platelet-endothelial cell interactions, calcein-AM loaded platelets were incubated with immobilized HUVECs cultured in gelatin coated clear 96-well plates (Verheul et al., 2000). Platelets were activated with 0.5U/ml thrombin in these assays. The amount of platelet adhesion was measured after removal of unbound platelets by washing. Calcein-AM fluorescence was measured as an indicator of the number of adhered platelets (Li et al., 1996). The time course of binding of activated platelets to HUVECs showed a significant increase in platelet binding only after 30 minutes and maximum binding occurred between 45 and 60 minutes after the start of the assay (Figure 4.13a). Endothelial cells or platelets pre-treated with Abciximab (20µg/ml) and RGD (150µg/ml) were significantly inhibited in their ability to interact (Figure 4.13b and c). The presence of the inhibitory antibody against VE-Cadherin (BV9) in both platelets and endothelial cells failed to block the platelet adhesion to endothelial cells (Figure 4.13c). This is suggesting that VE-Cadherin may not play role in platelet-endothelial interaction.

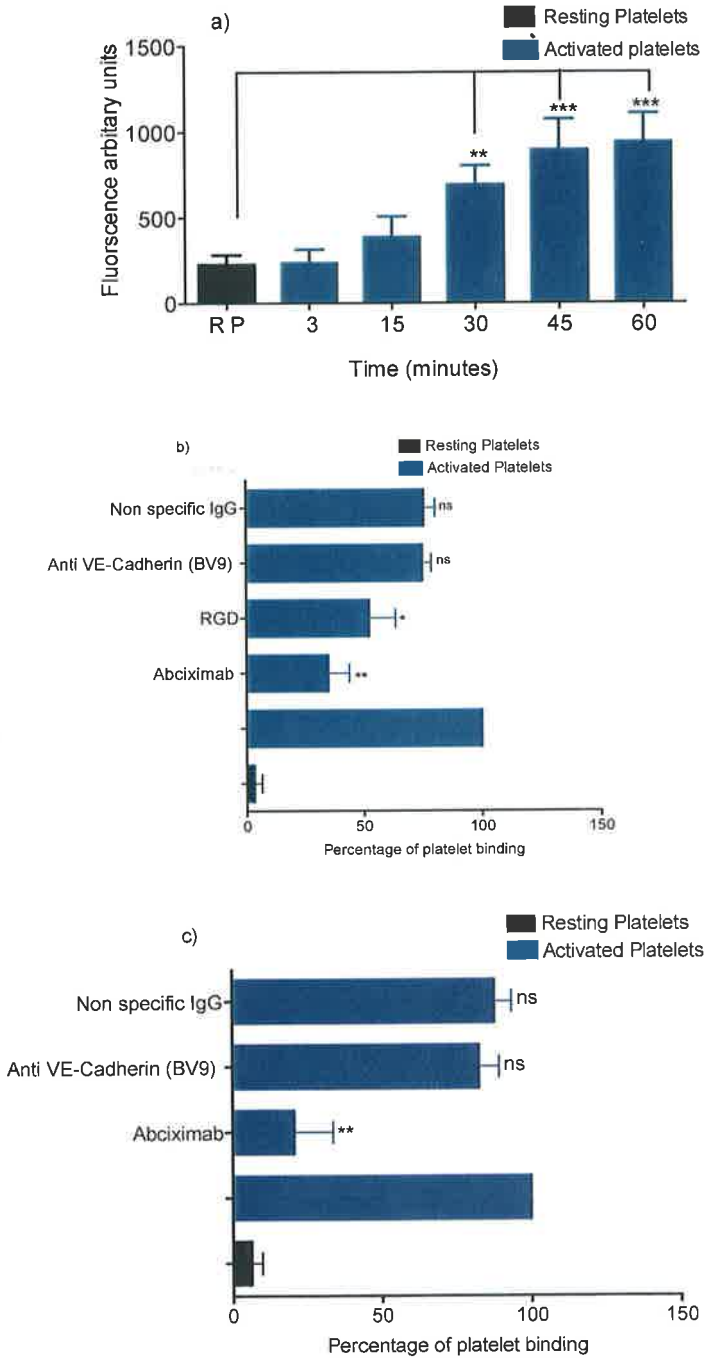
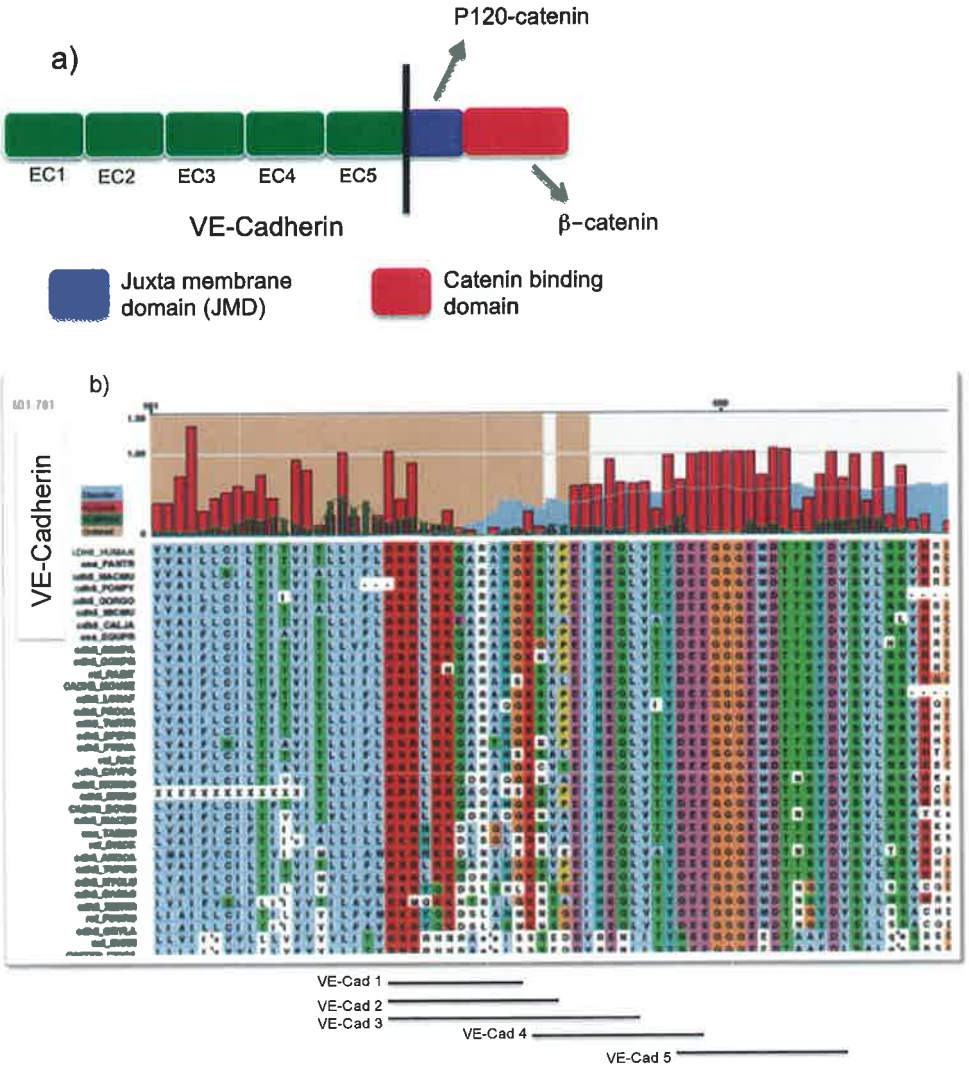


Figure 4.13 Adhesion of activated platelets to endothelial cells. For adhesion assay HUVECs were grown in 2% gelatin coated 96-well platelets in the absence of VEGF. Thrombin (0.5U/ml) treated platelets were allowed to adhere to HUVECs for 45 minutes. Platelet binding was determined as described in methods (section 2.2.14) using the calcein-AM method. a) Time course of platelet binding to endothelial cells. Data is expressed as the amount of platelets adhered, in calcein AM fluorescence arbitrary units. b) Adhesion of activated platelets to HUVEC after blockage of platelet receptors. c) Adhesion of activated platelets to HUVEC after blockage of HUVEC receptors. The following concentrations of antagonists were used; RGD (150µg/ml), Abciximab (20µg/ml) and all other antibodies (40µg/ml). Error bars represents mean ±SEM of N=4 individual experiments, where *P<0.05, **P<0.001 and ***P<0.0001 indicates significance measured by One-way ANOVA. Significance was compared with adhesion of activated platelets to HUVEC in the absence of any antagonist.

4.2.11 Design of VE-Cadherin peptides

According to the previous results, platelets do not express E- and N-Cadherins (Figure 4.1) but peptides derived from cytoplasmic portions of these cadherins inhibit the platelet function (Figure 3.6). However, those effects were identified as being mediated through non-specific inhibition (Figure 3.8). The interaction between the VE-Cadherin JMD and P120-catenin is crucial for cadherin regulation in other non-platelet cells (Iyer et al., 2004). Therefore the effects of peptides derived from the JMD of VE-Cadherin were analysed in platelet function to determine if such peptides could possibly help to determine a role for VE-Cadherin in platelets. VE-Cadherin peptides were designed using similar approach as described in chapter 3. Short linear motif prediction (SLIM) analysis suggested that the JMD region is highly conserved in its orthologous proteins (Figure 4.14b). Overlapping peptides from the JMD to the P120-catenin binding region (Figure 4.14c) were designed and synthesized with N-terminus palmitoylation, and analyzed in a platelet ATP secretion assay. These new peptides are similar to the ones described in chapter 3 and are designed to be analogous to those used in the analysis of the function of N- E- and K-Cadherin. As described previously (section 3.2.1), peptides derived from regions located at a distance from the membrane were modified with a Ttds linker (section 3.2.1) between the palmitate moiety and the active peptide. This is intended to mimic the physical distance from the membrane in VE-Cad 4 and 5 peptides. Randomly scrambled peptides were used as control peptides. The random scrambled peptides were designed using (http.//bioware.ucd.ie/~testing/biowareweb/) as described previously (section 3.2.6).



c)

Synonym	Parent Peptide	Control to parent Peptide
VE- Cad 1	RRRLRKQARAHG	RLQRARGRARHK
VE- Cad 2	RRRLRKQARAHGKSV	KKGLRSRAQHVRARR
VE- Cad 3	RRRLRKQARAHGKSVPEIHEQL	REKIRQAQSELHRAKRHRLGPV
VE- Cad 4	SVPEIHEQLVTYDEE	PSVDHVIELETQEEY
VE- Cad 5	DEEGGGEMDTTSYDV	TGDEMSDEYEVGDTG

Figure 4.14 Design of VE-Cadherin peptides. a) Graphical representation of VE-Cadherin 1-5 extracellular domains, juxtamembrane domain (JMD) and catenin binding domain (CBD). b) SLIM prediction analysis of human VE-Cadherin juxtamembrane domain. Relative local conservation (RLC) (red bars), disorder region (blue) and SLIM prediction (green bars). Alignment of VE-Cadherin with its orthologous is shown. Peptides derived region indicated at bottom of the alignment. Peptide synonyms, sequences and control peptide sequences were listed in table (c).

4.2.12 Assessment of VE-Cadherin derived peptides in platelet function

The effect of VE-Cadherin-derived peptides were analysed in the platelet ATP secretion assay in the presence and absence of various agonists TRAP, collagen and the thromboxane mimetic (U46619) (Figure 4.15). VE-Cadherin peptides inhibited TRAP-induced platelet function at 50μM (Figure 4.15a). In response to collagen, VE-Cad 1 to 3 peptides and their controls did not inhibit platelet function (Figure 4.15b). However, VE-Cad 4, 5 and their controls significantly inhibited collagen induced platelet response (Figure 4.15b); suggesting peptides might be acting in different pathways. Most of the peptides also attenuated the response of U46619 except VE-Cad 1 peptide (Figure 4.15c). VE-Cad 1 and its control peptide were able to induce the platelet activation but it did not interfere with Collagen or U46619 induced platelet activation (Figure 4.15). However, the toxicity results identified (both LDH and RBC lysis assay), VE-Cad 1 and its control peptide identified as toxic peptides in the lactate dehydrogenase (LDH) assay and RBC lysis assay (data not shown) respectively (Figure 4.17). The detection of ATP release with VE-Cad 1 and its control peptide is probably associated with partial damage to the platelet membrane. In addition, most of the control peptides mimic the effects of the parent peptides and also inhibited the agonist induced platelet activation. This result strongly suggests that the peptide effects observed at 50μM dose are non-specific since they are not sequence dependent. Therefore the effects of these peptides were subsequently assessed at lower doses to determine if more specific effects could be observed in the platelet ATP secretion assay. The potency of VE-Cadherin peptides analysed by incubation of platelets with various concentrations of peptides (0.8, 3 and

12.5μM) (Figure 4.16). This dose dependent analysis revealed that 12.5μM is the minimum concentration of VE-Cad 1,2 and 3 peptides that can significantly inhibit the platelets response to TRAP (Figure 4.16). Surprisingly control peptides to VE-Cad 1, 2 and 3 peptides also inhibited the TRAP induced platelet activation at 12.5μM. These results are clearly suggesting that anti-platelet activity of VE-Cadherin-derived peptides is similar the effects observed for N- and E-Cadherin-derived peptides as described in chapter 3.

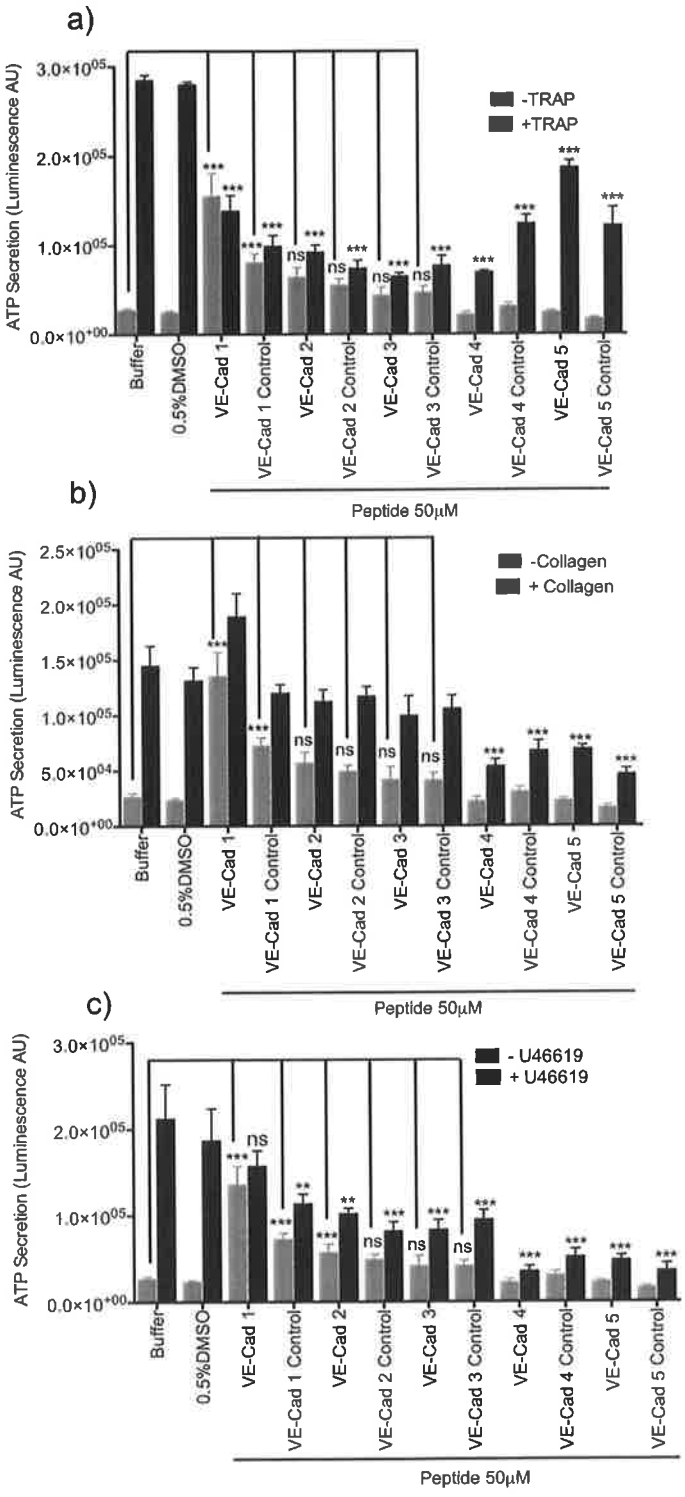


Figure 4.15 Effects of VE-Cadherin peptides on platelet function. 50μM concentration of each peptide was incubated with washed platelets for 12 minutes at 37°C followed by activation of platelets with TRAP (4μM) (a), collagen (38.5μg/ml) (b) and (2.5μM) of U46619 (c). Grey bars represent platelet response to peptides in the absence of agonist and black bars represent peptide along with the indicated agonist. Data shown is mean ±SEM of N=4 individual donors. **P<0.001 and ***P<0.0001 indicates significance calculated using One-way ANOVA. Significance was obtained by comparing the platelets ATP secretion to TRAP in the absence of any peptide and platelet ATP secretion to buffer in the absence of TRAP.

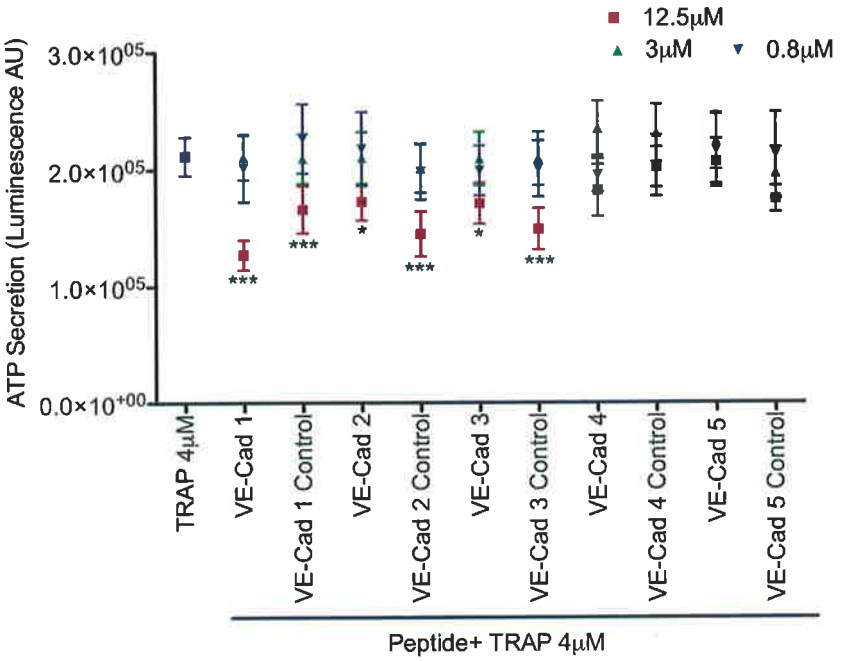


Figure 4.16 Effects of various concentrations of VE-Cadherin peptides on platelet function. Washed platelets were treated with various concentrations of VE-Cadherin peptides (0.8 3 and 12.5μM) for 12 minutes at 37°C and platelets were activated with standard dose of TRAP (4μM). Data shown is mean ±SEM of N=4 individual donors, where * P<0.05 and ***P<0.0001 represents significance calculated using One-way ANOVA. Significance was obtained by comparing the platelets ATP secretion to TRAP in the absence of any peptide.

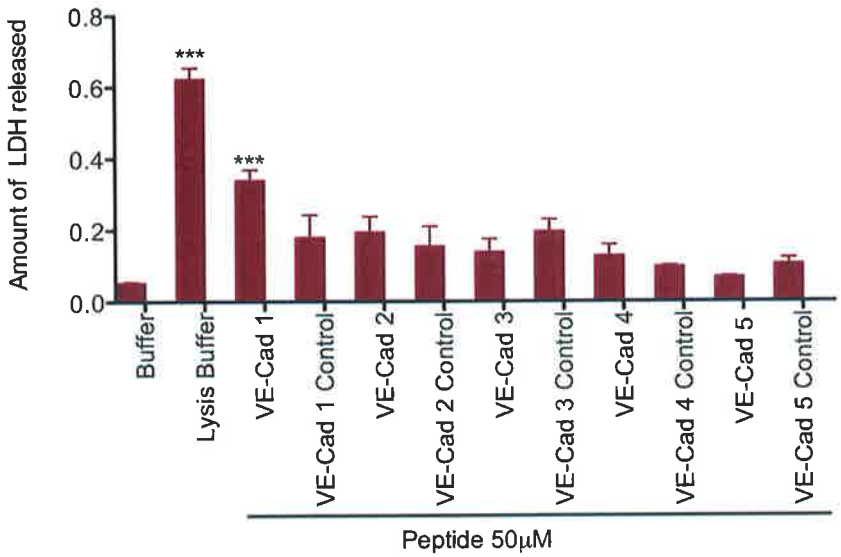


Figure 4.17 Toxicity of VE-Cadherin peptides on platelets. Washed platelets were treated with 50μM of VE-Cadherin peptides for 15 minutes at 37°C under agitation. After incubation samples were centrifuged at 720xg for 3 minutes and 50μl of supernatant was carefully transferred to clear 96-well plates. The amount of toxicity was measured as LDH enzyme in the supernatant from platelets using the LDH detection reagent as described in methods (section 2.2.5). Significance was compared to buffer treatment in the absence of any peptide. Data is presented as mean ±SEM of N=3 individual donors. *P<0.05, **P<0.001 and ***P<0.0001 indicates significance, calculated using One-way ANOVA.

4.3 Discussion

This present study identified a novel cell adhesion molecule (CAM) VE-Cadherin and its associated protein P120-catenin in human platelets using three different assays, western blot, flow cytometry and mass spectrometry. It was also established that platelet integrin αIIbβ3 can bind to VE-Cadherin in an RGD-dependent manner. This result was confirmed using CHO cells which express the platelet integrin αIIbβ3. Furthermore, findings in this chapter also demonstrate that the integrin activation status does not seem to affect the ability to bind to VE-Cadherin.

The expression of VE-Cadherin was confirmed in human platelets using the anti-human VE-Cadherin (clone 16B1) antibody. The antibody to VE-Cadherin was characterized using several approaches. In particular, western blotting of known VE-Cadherin expressing cells (HUVECs) were used to ensure that the antibody recognized a specific band in these cells. In parallel, expression of VE-Cadherin was also cross-checked in platelets using flow cytometry. Then cells that are known not to express VE-Cadherin (HK-2 and MBA MD-231) were used to demonstrate that the VE-Cadherin antibody did not cross-react with other cadherins. Finally, expression of VE-Cadherin was further confirmed by analysis of VE-Cadherin immunoprecipitates from platelets in mass spectrometric analysis. In parallel the expression of a previously identified CAM K-Cadherin (Dunne et al., 2012) was also verified using a K-Cadherin-specific antibody. It is noteworthy that no K-Cadherin was observed in the VE-Cadherin immunoprecipitated samples. Likewise, no VE-Cadherin was observed in the samples immunoprecipitated with the K-Cadherin

antibody. This demonstrates that each antibody was specific for only one cadherin. Elrod *et a*l. had previously shown the evidence for the expression of E-Cadherin in platelets (Elrod et al., 2003). However, there was no evidence for E-Cadherin either in western blots or in any of the mass spectrometric analysis of platelet cadherins in my studies. The specific antibodies used in Elord *et al.* study differed from those used in my study (Elrod et al., 2003). We used E-Cadherin antibody (clone 24E10) and demonstrated its sensitiviy using MCF7 cells which specifically express E-Cadherin. In general, classical cadherins share 20 to 30% sequence homology in their amino acid sequence (Nollet et al., 2000). Detection of E-Cadherin in Elord *et al.* study may be due to the binding of E-Cadherin antibody to common epitope within the other calssical cadherins (Elrod et al., 2003).

The bands of VE-Cadherin that were observed in the western blot migrated at a slightly lower molecular weight than the bands in positive control cell line (HUVEC). This might be due to different glycosylation of VE-Cadherin in platelets compared to endothelial cells. The evidence of VE-Cadherin glycosylation was obtained from Geyer and co-workers who demonstrated that VE-Cadherin carries predominantly sialylated complex and hybrid-type glycans (Geyer et al., 1999). Moreover, Brasch *et al.* mapped five N-linked glycosylation sites in the human VE-Cadherin EC 1 to 4 (Brasch et al., 2011). However, the aspect of platelet VE-Cadherin glycosylation was not explored further.

Classical cadherins such as VE-Cadherin are known to form interactions with cytoplasmic proteins called catenins. Therefore, platelet lysates were further screened for the presence of catenins. P120-catenin prevents the internalization of VE-Cadherin by protecting it from the clathrin-dependent endocytic pathway (Iyer et al., 2004, Chiasson et al., 2009). The association of β-catenin with VE-Cadherin supports the function of VE-Cadherin in endothelial cell permeability by forming a junction complex anchored to the actin cytoskeleton, promoting cell-cell adhesions and maintaining barrier function (Mehta and Malik, 2006). In contrast, in platelets, a role for β-catenin was identified as a key regulator of wnt-signaling (Steele et al., 2009). In this chapter, P120-catenin, but not either α-catenin or junctional plakoglobin was identified in human platelets. This causes us to speculate that P120-catenin might regulate the VE-Cadherin stability in platelets in a similar manner to its function in endothelial cells. However, we did not follow up on a functional role for P120-catenin and focused instead on attempting to identify a role for VE-Cadherin.

VE-Cadherin is one of the major proteins in endothelial cells. Activation of endothelial cells with growth factors such as, VEGF will induce the down regulation and phosphorylation of VE-Cadherin (Gavard et al., 2008, Esser et al., 1998). In this regard, analysis of expression and phosphorylation of VE-Cadherin upon platelet activation was analysed to determine if parallel behavior could be observed in platelets. However, VE-Cadherin did not down regulate nor phosphorylate upon platelet activation. These results suggest

that VE-Cadherin may not play a similar role in platelet activation as that observed in endothelial cells.

It is well established that platelet aggregation is mainly mediated by cross-linking of platelets via fibrinogen binding to platelet integrin. Recent work has shown that platelet aggregation was inhibited using a specific polyclonal antibody against the full length extracellular portion of K-Cadherin and suggested a role for cadherin in platelet aggregation (Dunne et al., 2012). In this chapter the role of VE-Cadherin in platelet aggregation was investigated using a monoclonal blocking antibody against VE-Cadherin (BV9). BV9 can specifically bind to the extracellular domain of VE-Cadherin and induce the vascular permeability and inhibit the angiogenesis by redistribution of VE-Cadherin at intracellular junctions (Corada et al., 2001). In our hands, blocking of platelet VE-Cadherin using BV9 did not inhibit the platelet aggregation. In addition, blocking of VE-Cadherin did not support or augment the inhibition of platelet aggregation in the presence of low concentrations of Abciximab. This is indicating that VE-Cadherin may not mediate the platelet-platelet interaction directly through cadherin-cadherin interaction.

Platelets can adhere to several proteins such as fibrinogen and VWF (Smyth et al., 2009). The interaction between fibrinogen and platelets is mediated by platelet integrin αIIbβ3. Dunne *et al.* have shown that platelets can adhere to K-Cadherin via αIIbβ3 in a RGD dependent manner (Dunne et al., 2012). This chapter demonstrates that platelets can also adhere to immobilized VE-Cadherin and their interaction is inhibited in the presence of RGD peptides.

This observation suggested that VE-Cadherin, like K-Cadherin, may bind to integrin αIIbβ3 in a RGD dependent manner. However, RGD peptides can bind not only to platelet integrin αIIbβ3 but also to several other integrins including αvβ3, αvβ5 and αvβ1 (Thibault et al., 2001). The inhibition of platelet adhesion to immobilized VE-Cadherin by RGD peptides may be suggestive of a role for any integrin in this process and not specifically a role for αIIbβ3. To answer this question of whether αIIbβ3 is important in this adhesion, we have used abciximab, which specifically blocks the function of just two integrins, αIIbβ3 or αvβ3. This agent also inhibited platelet adhesion to VE-Cadherin. In addition, CHO cells expressing integrin αIIbβ3 adhered to VE-Cadherin. Together, this suggests that a direct interaction between immobilized VE-Cadherin and αIIbβ3 exists.

It is noteworthy that CHO cells expressing a mutant form of integrin αIIbβ3, which is in a constitutively active conformation (αIIbβ3AA), is equally able to bind to VE-Cadherin. This shows that the cadherin-integrin interaction was independent of integrin activation status. This raises the question of whether VE-Cadherin on resting platelets might bind to adjacent resting platelets (via αIIbβ3) or to endothelial cells (via αvβ3) in intact blood vessels. To answer this question, I explored the ability of BV9, a known inhibitor of VE-Cadherin, to inhibit platelet aggregation and platelet adhesion to endothelial cells. BV9 had no effect on either of these assays. Thus it seems that platelet VE-Cadherin is not mediating a physiological response in these situations. However, it may be that in conditions like vascular stress or injury, platelet VE-Cadherin adopts a novel role. In a similar manner, under physiological

conditions, platelet integrin αIIbβ3 does not bind to soluble fibrinogen but can bind to immobilized fibrinogen (Bennett, 2005). Based on these observations we propose that VE-Cadherin on platelets may have a unique role in special pathological conditions. Elucidation of the precise conditions will require further experiments.

The interaction of activated platelets with endothelial cells is crucial for hemostasis. Various extracellular matrix proteins and platelet adhesion receptors support the interaction of platelets and endothelial cells (Bombeli et al., 1998). The interaction of activated platelets with endothelial cells is known to be mediated by integrins via fibrinogen (Bombeli et al., 1998). The role of VE-Cadherin on platelets was further assessed in platelet-endothelial cell interactions. In this chapter, blocking of VE-Cadherin either on platelets or endothelial cells did not influence the platelet-endothelial cell interactions suggesting that VE-Cadherin may not play any role in platelet-endothelial cell interactions. Based on platelet adhesion to endothelial cells and platelet adhesion to VE-Cadherin, it was concluded that platelet integrin αIIbβ3 might interact with VE-Cadherin on endothelial cells. The role of platelet VE-Cadherin and endothelial VE-Cadherin in platelet-endothelial cell interaction is still unknown. The platelet VE-Cadherin could bind to the endothelial integrin αvβ3. Using an antibody against VE-Cadherin (BV9) in both platelets and endothelial cells did not reveal any further clarification in the role of VE-Cadherin in platelet-endothelial cell interaction. The accurate blocking region of BV9 antibody in VE-Cadherin is still unclear. Breviario *et al.* demonstrated that blocking of VE-Cadherin using three different antibodies, which bind to

various motifs of VE-Cadherin extracellular domain, did not inhibit the VE-Cadherin mediated cell aggregation (Breviario et al., 1995). This suggests that VE-Cadherin homotypic binding is due to the multiple interactions of different molecular epitopes (Corada et al., 2001). In this case different binding sites needed to be blocked at the same time. One monoclonal antibody or even the cocktail of the three might not be sufficient for this purpose. Alternatively the ideal ways to explore the role of VE-Cadherin in platelet/endothelial cell interaction is by using an inhibitory polyclonal antibody or cell-models containing knock down of VE-Cadherin in endothelial cells using Small interfering RNA (siRNA).

In my study, the role of VE-Cadherin in platelets was further explored using peptides derived from JMD of VE-Cadherin. In similar to peptides used chapter 3, the effect of VE-Cadherin-derived peptides on platelets was also identified as non-specific. VE-Cadherin is a key player of several functions in endothelial cells. Therefore, we explored if peptides derived from VE-Cadherin JMD might be useful to explore their effects in vascular endothelial cells.

Initially the aim of this chapter was to characterize the expression of cadherins in human platelets. In summary, it was concluded that, human platelets express novel cell adhesion molecule (CAM) VE-Cadherin and its associated protein P120-catenin. In addition, this study established that VE-Cadherin is a novel ligand of platelet integrin αIIbβ3. Further analysis of the role of VE-

Cadherin in platelets might reveal physiological functions of this protein in hemostasis.

Chapter 5

Assessment of VE-Cadherin derived Peptides in Endothelial Cell Function

5.1 Introduction

Angiogenesis is a multi-step process that begins with endothelial cell (EDC) permeability, migration, breakdown of basal membrane, followed by EDC proliferation, EDC migration and finally formation of stable blood vessel (Nussenbaum and Herman, 2010). This complex process involves a number of EDC receptors and signaling pathways. Under normal physiological conditions, angiogenesis occurs at sites of vascular injury and normal vessel growth. However, it is becoming increasingly apparent that angiogenesis also occurs under pathophysiological conditions and is important in the progression of certain disease states, most notably cancer (Coultas et al., 2005). For example, in order for a tumour to grow larger than $1mm^3$, it must develop its own blood supply, highlighting the importance of angiogenesis in tumour growth. As such, inhibitors of angiogenesis could be extremely useful in the treatment of cancer. Folkman *et al.* first observed the inhibition of tumour growth in the absence of neovascularization (Folkman et al., 1963). Further work on inhibition of blood vessel growth has shown greater impact on cancer therapy (Ribatti, 2008).

VE-Cadherin plays a crucial role in angiogenesis. Homophilic interaction between VE-Cadherins, support the endothelial cell stability. VE-Cadherin importance in angiogenesis has been clearly demonstrated using VE-Cadherin null mice and functional blocking monoclonal antibodies (Liao et al., 2000). VE-Cadherin null embryos exhibited nascent vessels growth. However, these embryos failed to stabilize the nascent vessels due to lack of VE-Cadherin (Crosby et al., 2005). VE-Cadherin does not regulate vascular

patterning but it is important in vascular connections and inhibition of cell sprouting. These processes require stable cell-cell junctions which is lacking in the absence of VE-Cadherin (Giannotta et al., 2013). The partial internalization or changes in VE-Cadherin function may also greatly affect the vascular permeability (Giannotta et al., 2013). Endothelial cell growth is balanced by crosstalk between intracellular domains of VE-Cadherin and Vascular endothelial growth factor receptor 2 (VEGFR2), which requires the association of β-catenin with VE-Cadherin (Dejana, 2004).

Several blocking antibodies that target the extracellular region of VE-Cadherin have been used to study its role in angiogenesis (Liao et al., 2000, Corada et al., 2002). These antibodies are mostly custom-synthesized antibodies used by individual laboratories. BV9 is one of the inhibitory antibodies that binds to extracellular domains of VE-Cadherin and inhibits its function by redistribution (Corada et al., 2002). However, the role of the cytoplasmic domain of VE-Cadherin in angiogenesis has not been investigated. Previously, it has been demonstrated that peptides derived from crucial regions of other endothelial cell receptors (e.g. integrin αvβ3) involved in angiogenesis have anti-angiogenic properties (Foubert and Varner, 2012, Garcia-Aranda et al., 2013, Goncalves et al., 2007, Mas-Moruno et al., 2010).

Therefore, in this chapter, the effect of cell permeable peptides derived from the JMD of VE-Cadherin on endothelial cell function and angiogenesis was investigated. The peptides were designed using computational methods as described in previous chapters 3 and 4. Previously, peptides showed effects

that were eventually deemed to be of a mainly non-specific nature, it was considered worthwhile to reassess the effects of VE-Cadherin peptides in an endothelial system where the function of VE-Cadherin is well established. In these studies, it was decided that the dose of 50μM, utilized extensively in the initial studies, was probably too high. Therefore lower doses of peptides were utilized to understand VE-Cadherin peptides effects in endothelial cells. In addition, because VE-Cad 1 peptide was determined to be toxic in the LDH toxicity assay (Figure 4.17), it was omitted from this chapter. Using various assays (capillary tubule formation assay, scratch wound assay, and proliferation assay) the effect of VE-Cadherin peptides was explored.

5.2 Results

5.2.1 Anti-angiogenic activity of VE-Cadherin peptides

To examine the effect of VE-Cadherin JMD-derived peptides on angiogenesis, human umbilical vein endothelial cells (HUVECs) were treated with low (5μM) and high (20μM) in the *in vitro* assay of endothelial cell function, angiogenesis. Similar to chapter 4, VE-Cadherin, 2, 4 and 5, and their corresponding control peptides were tested. The VE-Cadherin functional blocking monoclonal antibody BV9 (40μg/ml) was used as a positive control for angiogenesis inhibition (Corada et al., 2002).

For angiogenesis assay the wells of a 96-well plate, were coated with Matrigel (a gelatinous protein mixture that mimics the basal membrane; it consists of laminin, collagen type IV, heparin sulfate proteoglycans, entactin and nidogen), HUVECs were seeded onto the Matrigel and allowed to form

capillary tube networks in the presence of VEGF (10ng/ml) for 12 hours. In buffer treated (cells alone) wells, HUVECs formed large complex networks of capillary tubules (Figure 5.1). As expected, the VE-Cadherin blocking antibody (BV9) decreased the number and length of capillary tube formation response to 70 to 80% compared to buffer treated wells (cells alone) (Figure 5.2). Interestingly, in the presence of VE-Cad 2 or VE-Cad 4 peptides, there was a significant decrease in capillary tubule formation and capillary tubule length. This inhibition was dose dependent and a greater effect was observed with 20μM than with 5μM peptide (Figure 5.1 and 5.2). For example, in the presence of VE-Cad 2 and 4 peptides at 5μM and 20μM, the number of tubules and tubule length was reduced to 50 to 60% and 80 to 90% respectively compared to buffer treated cells (cells alone) (Figure 5.1 and 5.2). Interestingly VE-Cad 2 peptide at 20μM was more significantly inhibited the tubule length compare to positive control (BV9) (Figure 5.1 and 5.2).

Interestingly, the VE-Cad 2 and 4 control peptides did not decrease capillary tubule formation or tubule length, at both 5 and 20μM concentrations (Figure 5.1 and 5.2). This is indicating that the VE-Cad 2 and 4 peptides have specific anti-angiogenic properties. In contrast, VE-Cad 5 displayed no anti-angiogenic properties at either 5 or 20μM (Figure 5.1 and 5.2). However, control peptide to VE-Cad 5 did show some significant inhibition of tubule formation at 20μM (Figure 5.2b).

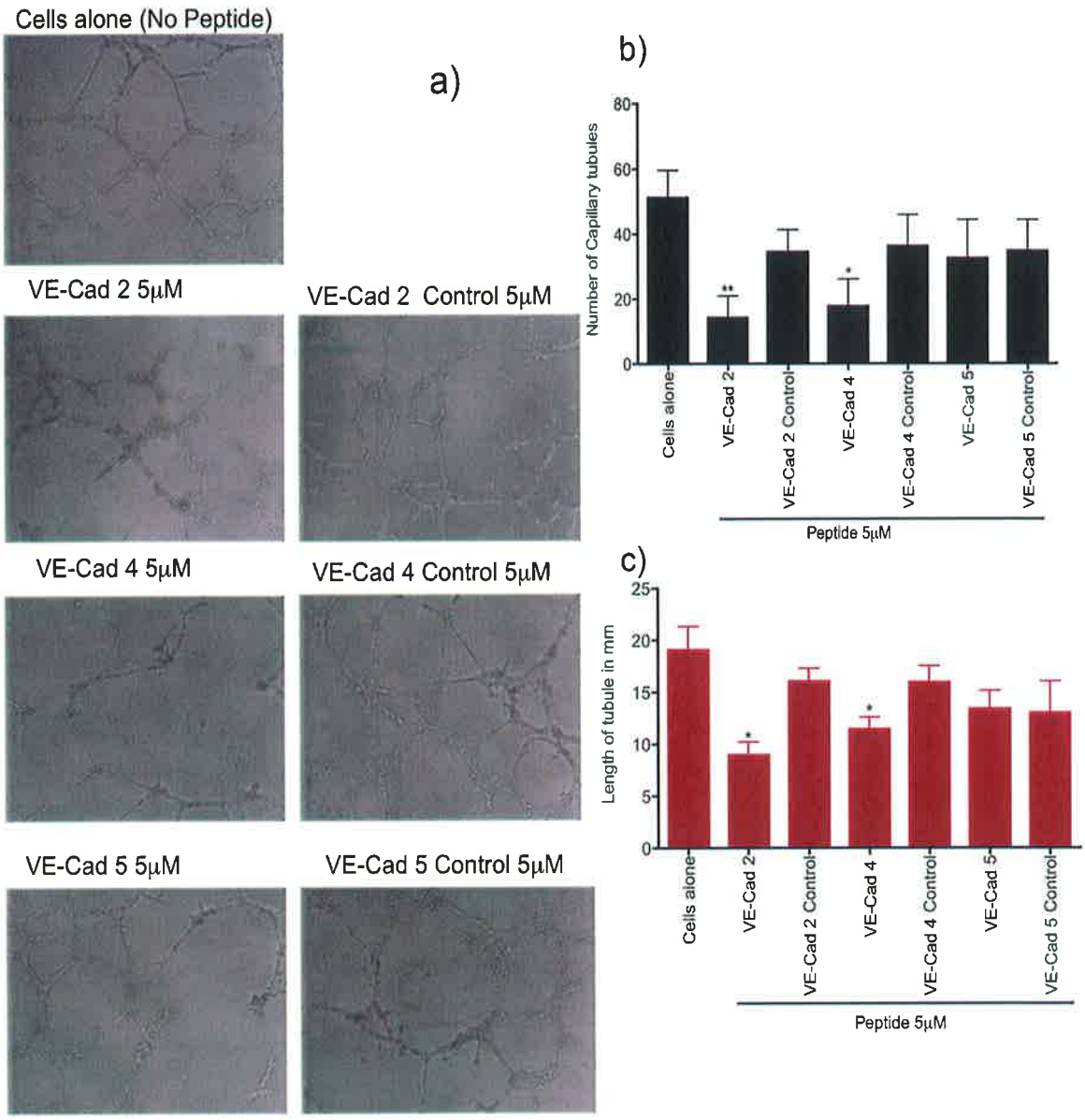


Figure 5.1 VE-Cadherin JMD derived peptides (5μM) specifically inhibit angiogenesis, as measured by capillary tube formation. Clear 96-well plates were coated with Matrigel as described in methods (section 2.2.11). HUVECs ($2X10^4$) were seeded into each well in the absence (cells alone) or presence of VE Cadherin derived peptides and their appropriate control peptides (5μM). HUVECs were allowed to form capillary tubules for 12 hours at 37°C. After 12 hours, three DIC images (10 x) were taken per each well. From these images, the number of capillary tubules formed was counted manually and the length of each tubule (in mm) was calculated using ImageJ. a) Representative images of capillary tubule formation by HUVECs in the absence (cells alone) and presence of VE-Cadherin peptides along with their appropriate controls. b) Histogram plot showing the effect of VE-Cadherin peptides and their corresponding controls (5μM) on number of capillary tubule formation in HUVECs. c) Histogram plot showing the effect of VE-Cadherin peptides and their corresponding controls (5μM) on the length of capillary tubules formed by HUVECs. Data was analysed using One-way ANOVA of N=3 individual experiments. The effect of the peptides was compared to the cells alone control (* $P<0.05$, ** $P<0.001$ and *** $P<0.0001$). Scale bar for each picture =100μm.

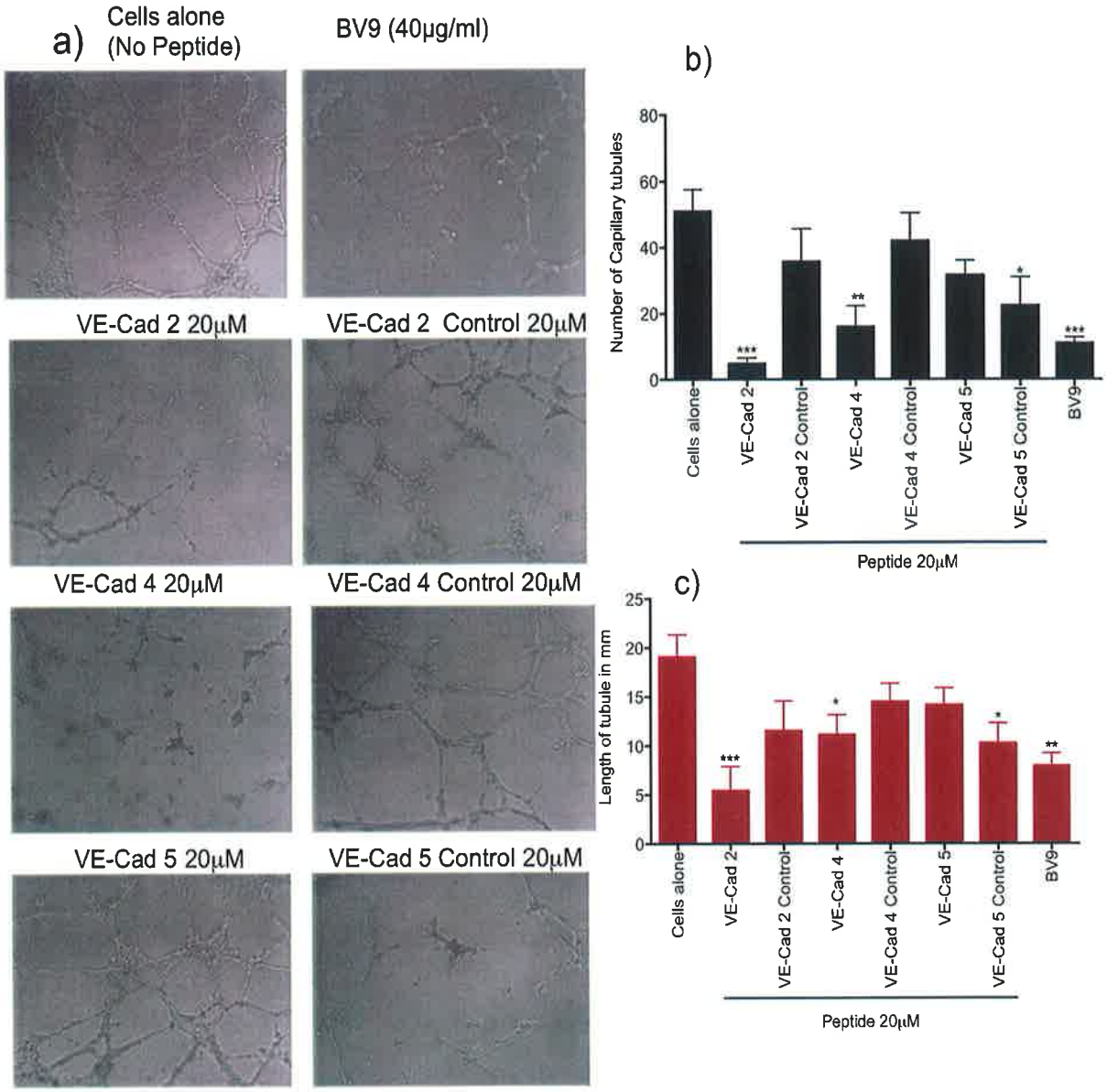


Figure 5.2 VE-Cadherin derived peptides at high concentration (20µM) specifically inhibit angiogenesis, as measured by capillary tube formation. Clear 96-well plates were coated with Matrigel as described in methods (section 2.2.11). HUVECs ($2X10^4$) were seeded into each well containing HUVEC media in the absence (cells alone) or presence of VE-Cadherin peptides and their appropriate control peptides (20µM). The anti VE-Cadherin monoclonal antibody BV9 (40µg/ml) was used as a positive control for inhibition of capillary tubule formation. HUVECs were allowed to form capillary tubules for 12 hours at 37°C. After 12 hours, three DIC images (10 x) were taken per each well. From these images, the number of capillary tubules formed counted manually and the length of each tubule (in mm) was calculated using ImageJ. a) Representative images of capillary tubule formation by HUVECs in the absence (cells alone) and presence of VE-Cadherin peptides along with their appropriate controls. b) Histogram plot showing the effect of VE-Cadherin peptides and their corresponding controls (20µM) on number of capillary tubule formation in HUVECs. c) Histogram plot showing the effect of VE-Cadherin peptides and their corresponding controls (20µM) on the length of capillary tubules (mm) formed by HUVECs. Data was analysed using One-way ANOVA of N=3 individual experiments. The effect of the peptides was compared to the cells alone control (* $P<0.05$, ** $P<0.001$ and *** $P<0.0001$). Scale bar for each picture =100µm.

5.2.2 VE-Cadherin peptides inhibited VEGF induced cell migration

Next, the effect of VE-Cadherin peptides were tested in a second endothelial function assay, the scratch wound healing assay, in order to confirm their biological effects. For this assay, HUVECs were grown in the wells of a 12-well plate. When confluent, a scratch or wound was made across the confluent HUVEC layer. The ability of endothelial cells to migrate and heal the wound area in the presence of VEGF (10ng/ml) was then measured.

In untreated control wells, endothelial cells migrated and grew into the wound area after 12 hours. The number of migrated cells was ≈60 per field in buffer treated wells (cells alone). However, in the presence of high concentrations of VE-Cad 2 & 4 JMD-derived peptides (20μM), the number of migrated cells decreased significantly (Figure 5.4). For example in the presence of VE-Cad 2 peptide their number was decreased down to 8 ($P<0.0001$) (Figure 5.4). Again, this inhibitory effect was specific since their corresponding control peptides had a no significant effect on endothelial cell migration (Figure 5.4). The inhibitory effect of VE-Cad 2 peptide but not 4 was also observed at the lower 5μM concentration (Figure 5.3) indicating that VE-Cad 2 is a more potent peptide than VE-Cad 4 peptide.

Similar to the results of the capillary tube formation assay, neither VE-Cad 5 peptide, nor its control peptide, had any effect on migration at both 5 and 20μM. Moreover control peptide to VE-Cad 5 had no significant effect at 20μM in this assay (Figure 5.3 and 5.4).

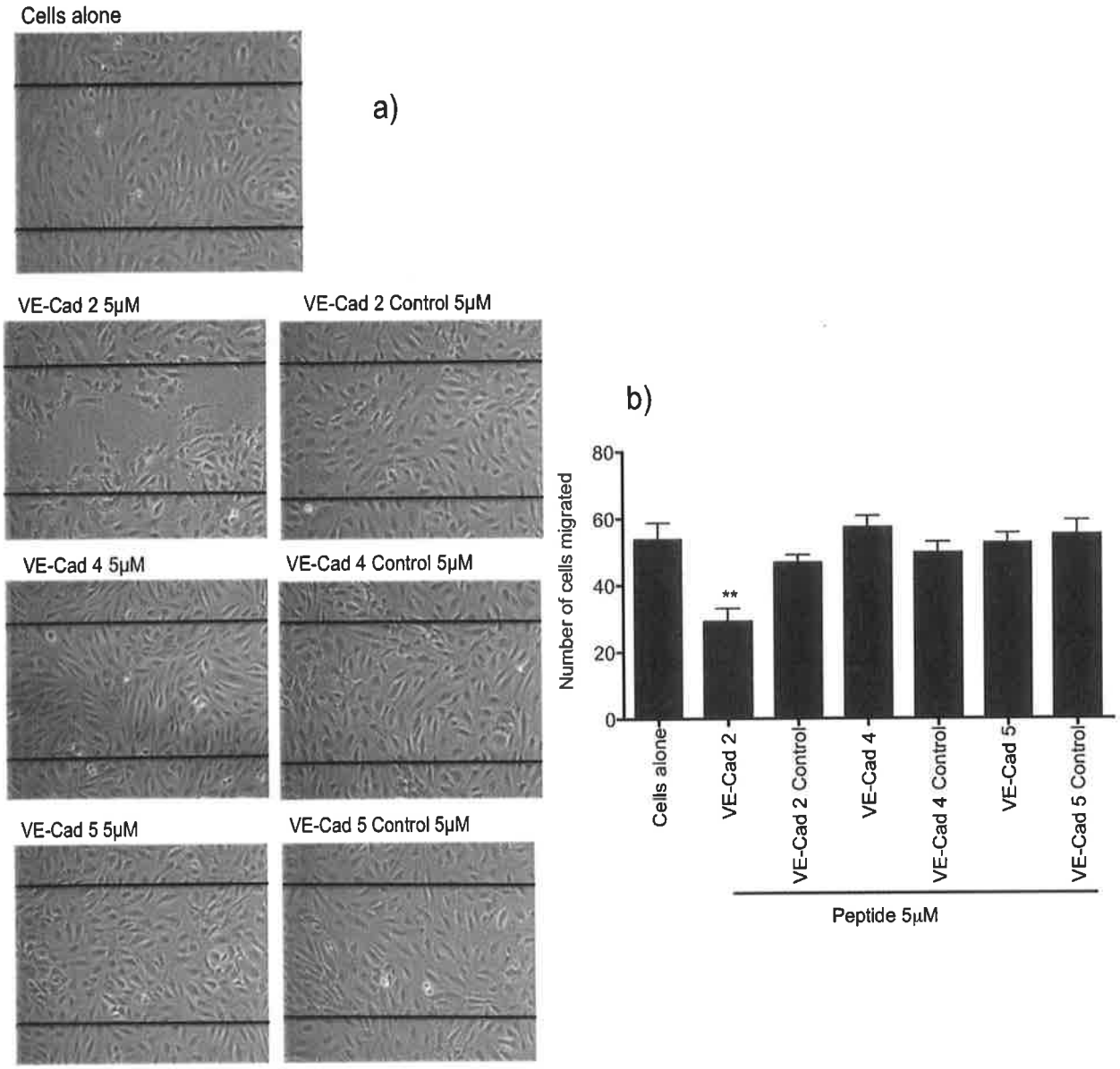


Figure 5.3 Effect of VE-Cadherin peptides (5μM) on endothelial cell migration a (wound healing) assay. For wound healing assay HUVECs were grown in 12-well plates until confluent. After overnight serum starvation a scratch was made across each well with a sterile pipette tip. The wells were then washed with PBS to remove unbound cells. Following this, HUVECs were incubated with HUVEC media containing VEGF (10ng/ml), in the absence (buffer) or presence of a low concentration of VE Cadherin peptides and their corresponding controls (5μM). Cells were allowed to migrate and heal the wound for 12 hours at 37°C. After 12 hours, three DIC images (10 x) were taken from random wound areas. From these images, the number of HUVECs that migrated into the wound area was calculated using ImageJ software. a) Representative images of the wound area of HUVECs with no peptide or VE-Cadherin peptides and their appropriate controls (5μM). b) Histogram plot showing the effect of VE Cadherin peptides or their corresponding controls (5μM) on HUVEC migration. Data was analysed using One-way ANOVA of mean ±SEM N=3 individual experiments. The effect of the peptides was compared to the buffer control (**P<0.001). Scale bar for each picture =50μm.

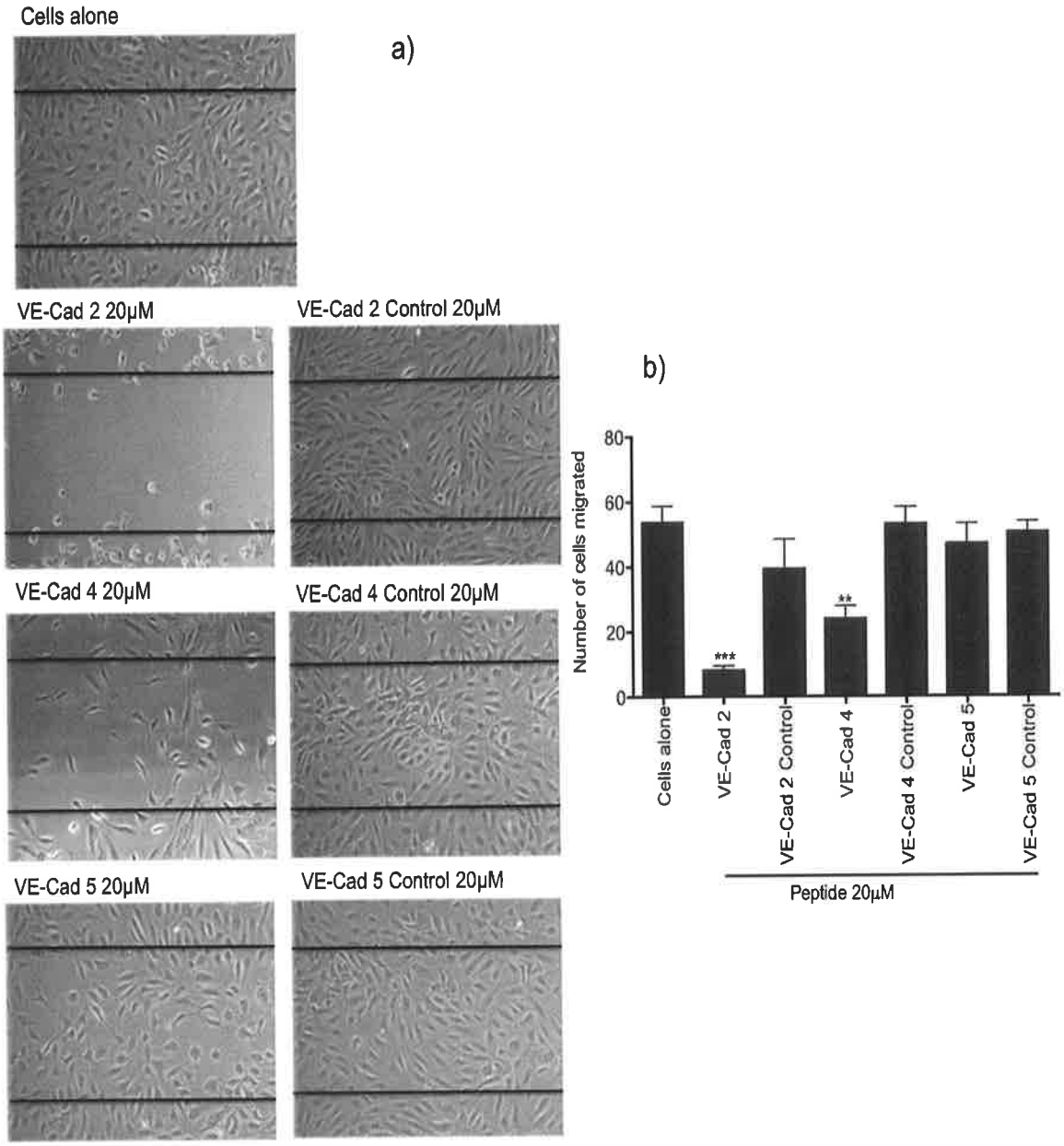


Figure 5.4 Effect of VE-Cadherin JMD-derived peptides (20μM) on endothelial cell migration (wound healing). HUVECs were grown in 12-well plates until confluent. Cells were serum starved overnight. After overnight serum starvation a scratch was made across each well with a sterile pipette tip. The wells were then washed with PBS to remove unbound cells. Following this, HUVECs were incubated with HUVEC media containing VEGF (10ng/ml), in the absence (buffer) or presence of a high concentration of VE-Cadherin peptides and their corresponding controls (20μM). Cells were allowed to migrate and heal the wound for 12 hours at 37°C. After 12 hours, three DIC images (10 x) were taken from random wound areas. From these images, the number of HUVECs that migrated into the wound area was calculated using ImageJ software. a) Representative images of the wound area of HUVECs with no peptide or VE Cadherin peptides and their appropriate controls (20μM). b) Histogram plot showing the effect of VE Cadherin peptides and their corresponding controls (20μM) on HUVEC migration. Data was analysed using One-way ANOVA of mean ±SEM N=3 individual experiments. The effect of the peptides was compared to the buffer control (**P<0.001 and *** P<0.0001). Scale bar for each picture =50μm

5.2.3 Effect of VE-Cadherin peptides on endothelial cell proliferation

Having established that VE-Cad 2 & 4 peptides inhibit endothelial cell function, as measured by capillary tube formation and migration, their effects on endothelial cell proliferation were next tested. Using the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, the effect of VE-Cadherin peptides on endothelial cell proliferation was investigated to observe whether the anti-angiogenic activity is associated with inhibition of cell proliferation or not. HUVECs were incubated with high or low concentrations of the peptides and proliferation was measured at 24 and 48 hours.

VE-Cad 2 peptide inhibited endothelial cell proliferation to 60 to 80% (0.128 and 0.092, $P<0.0001$) at 5 and 20μM respectively (Figure 5.5) compared to cells alone (0.389) after 48 hours of incubation. In addition, VE-Cad 4 peptide inhibited cell proliferation (0.122, $P<0.001$) only at 20μM compared to untreated cells (Figure 5.5a). Again, this inhibitory effect on endothelial cell proliferation was specific, since the corresponding control peptides did not inhibit endothelial cell proliferation (Figure 5.5).

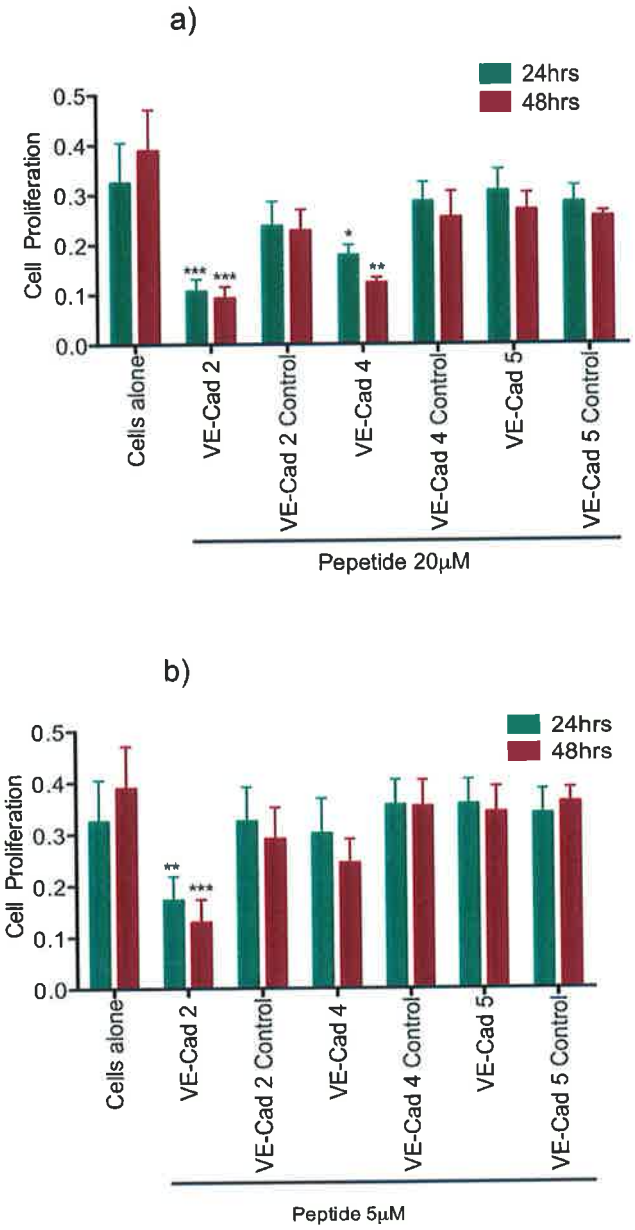


Figure 5.5 VE-Cadherin peptides 2 & 4 inhibit endothelial cell proliferation. The effect of VE-Cadherin peptides on endothelial cell proliferation was studied using MTS assay. HUVECs ($5X10^3$) seeded were into VEGF (10ng/ml) containing media in clear 48-well plates for 24 hours (green) or 48 hours (red) in the presence and absence of peptides. Following these time periods, the MTS assay was performed and cell proliferation was calculated. Each condition was assessed in duplicate wells. a) Histogram plot showing the effect of a high concentration (20μM) of VE-Cadherin peptides and appropriate control peptides on HUVEC proliferation at 24 and 48 hours. b) Histogram plot showing the effect of a low concentration (5μM) of VE-Cadherin derived peptides and appropriate control peptides on HUVEC proliferation at 24 and 48 hours. Data represents mean ±SEM of N=3 individual experiments. Significance was obtained by cell proliferation in the presence of peptides was compared to VEGF control using One-way ANOVA where, *P<0.05, **P<0.001 and ***P<0.0001.

5.2.4 Effect of peptides on VE-Cadherin expression in endothelial cells

VE-Cadherin peptides specifically inhibited the endothelial cell function. Their effect could potentially interfere with native VE-Cadherin-P120-catenin interactions or alter VE-Cadherin expression on endothelial cells. To investigate this hypothesis, the expression of VE-Cadherin levels in HUVECs after treatment with VE-Cadherin peptides was measured. Following serum starvation, cells were treated with 20μM of VE-Cad 2, 4 and their control peptides for 12 hours followed by activation of cells with 10ng/ml VEGF for 1 hour. After this, HUVECs were lysed with lysis buffer, samples were resolved using SDS-PAGE and blots were probed with an anti-VE-Cadherin antibody. In comparison to untreated HUVECs, there was no change in VE-Cadherin expression observed following treatment with VE-Cadherin peptides (Figure 5.6a), measured by densitometry analysis (Figure 5.6b). Equal protein loading in western blots was crosschecked by reprobing of anti-VE-Cadherin blots for β-actin (Figure 5.6a). This suggests that the peptides do not alter VE-Cadherin expression and that their anti-angiogenic activity is associated with mechanism other than decreased receptor expression.

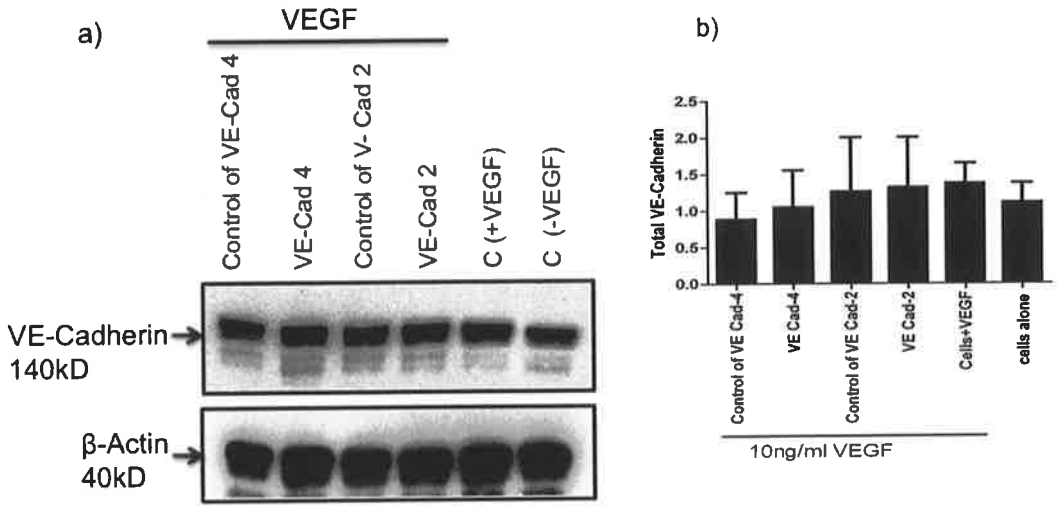


Figure 5.6 VE-Cad 2 & 4 peptides do not effect VE Cadherin expression on endothelial cells. HUVECs were grown in 6-well plates until confluent. Following serum starvation, cells were treated with 20μM of VE Cad-2, 4 and their control peptides for 12 hours followed by activation of cells with 10ng/ml VEGF for 1 hour. After 1 hour treatment with VEGF, HUVECs were lysed with lysis buffer, samples were resolved using SDS-PAGE and blots were probed with an anti-VE-Cadherin antibody. a) Expression of VE-Cadherin was examined by western blot analysis (upper panel). Cells, in the presence and absence of VEGF, were used as controls for VE-Cadherin expression. β-Actin was used as a loading control (lower panel). b) Densitometric analysis of total VE-Cadherin in untreated and treated HUVECs was calculated using area of cadherin density (determined by ImageJ analysis) divided by actin density. Data shown is mean ±SEM of N=3 individual experiments.

5.3 Discussion

In this chapter, a role of VE-Cadherin JMD in endothelial function was identified. Peptides derived from the JMD of VE-Cadherin significantly inhibited *in vitro* angiogenesis, cell migration and proliferation at 5 and 20μM. The specificity of the peptides was demonstrated using corresponding control peptides that were shown to have no effect on endothelial cell function or angiogenesis.

Angiogenesis is a key step in several pathological conditions like tumour growth. There are several receptors that can regulate angiogenesis. Identifying the regions of functional receptors that mediate their angiogenic properties could potentially aid the development of anti-angiogenic compounds that could target the tumour growth in cancer (Folkman et al., 1963). Previously, peptides derived from endothelial cell growth factors, such as VEGF and endothelial cell integrin αvβ3 have been shown to have anti-angiogenic properties (Garcia-Aranda et al., 2013, Goncalves et al., 2007, Mas-Moruno et al., 2010). A peptide derived from integrin αvβ3 (RGD) can selectively target the endothelial function by inhibition of integrin interaction with extracellular matrix proteins. The effect of this RGD peptide was also studied in clinical trials (Mas-Moruno et al., 2010). Moreover, studies suggested that identification of peptides derived from proteins involved in angiogenesis using computational approach is an ideal method to develop anti-angiogenic compounds (Rosca et al., 2011).

VE-Cadherin is a key player in angiogenesis. A number of blocking antibodies have been developed to target the adhesive nature of VE-Cadherin during angiogenesis (Corada et al., 2001, Corada et al., 2002). However, no compounds have been developed to target key regions of the cytoplasmic portion of VE-Cadherin.

Several studies have shown that JMD of VE-Cadherin is important in mediating interactions between VE-Cadherin and P120-catenein (Iyer et al., 2004). Ferber *et al.* identified that the octapeptide (YDEEGGGE) within the JMD of VE-Cadherin is crucial for P120-catenin binding and endothelial cell proliferation (Ferber et al., 2002). Therefore, in this chapter I focused on developing peptides corresponding to the potentially bioactive region of VE-Cadherin JMD. While these peptides failed to show any specific effects on platelet function in chapter 4. Using various endothelial cell functional assays, it has been shown that peptides derived from the JMD of VE-Cadherin can inhibit the endothelial cell function (migration and proliferation) and angiogenesis (capillary tube formation) at 5 and 20μM. These doses are lower than doses used in chapter 4. The VE-Cad 2 peptide was found to be a significantly more potent anti-angiogenic compound than both VE-Cad 4 and VE-Cad 5 peptides. The specificity of the VE-Cad 2 anti-angiogenic effect was verified using corresponding control peptide that was shown to have no inhibitory effect on endothelial cell function. These results suggest that peptides derived from JMD of VE-Cadherin can selectively inhibit the endothelial cell function.

It was hypothesized that targeting the JMD of VE-Cadherin might cause the degradation of VE-Cadherin in endothelial cells resulting in decreased surface expression of VE-Cadherin on endothelial cells. Therefore, the expression of VE-Cadherin in endothelial cells in the presence and absence of peptides was measured. Expression of VE-Cadherin did not alter in the presence of peptides relative to untreated endothelial cells. This suggests that the anti-angiogenic activity of VE-Cadherin peptides may not be due to decreased VE-Cadherin expression in endothelial cells. However, it is possible that the time course of the assay was simply not long enough to record any difference.

The mechanism of action of anti-angiogenic effect of VE-Cadherin peptides is still unknown. VE-Cadherin play important role in cell proliferation, the endothelial cell proliferation is mainly regulated by croostalk between VE-Cadherin and VEGFR2 (Dejana, 2004). The cytoplasmic region of VE-Cadherin binds to several cytoplasmic proteins such as catenins, and signaling molecules like C-terminal Src kinase (Csk) and density enhanced protein-1 (DEP1) (Dejana, 2004). The VE-Cadherin cytoplasmic domain associates with phosphatidylinositol-3-OH-kinase (PI3K)-AKT-Forkhead-box protein-O1 (FoxO1) pathway and reduces β-catenin transcriptional activity (Giampietro et al., 2012). In particular, the JMD of VE-Cadherin plays a crucial role in binding to P120-catenin (Iyer et al., 2004). In this current study, VE-Cadherin JMD peptides might be targeting the one of the key signaling mechanism in endothelial cells. However, in our studies, the peptides did not appear to destabilize cadherin expression, as would be expected if the peptides were actively competing with P120-catenin for binding to VE-

Cadherin JMD (Iyer et al., 2004). Further experimentation to determine if the experiments presented in this chapter were performed under optimal conditions is required.

In conclusion, it was demonstrated that peptides derived from the JMD of VE-Cadherin specifically inhibit angiogenesis, migration and proliferation without affecting VE-Cadherin expression in endothelial cells. These findings highlight the importance of the JMD of VE-Cadherin in endothelial cell function. Further investigations are required to understand the signaling mechanism of these peptides. In addition, this peptide design can be used as template for design of anti-angiogenic compounds from key proteins involved in endothelial cell function to target pathological angiogenesis.

Chapter 6

Prediction of different Cadherin-derived Peptide variables that can influence the Peptide Bioactivity

6.1 Introduction

Peptides are the small polymers of amino acids that can selectively modulate the functions of various proteins. They have been used in various therapeutic applications (Craik et al., 2013). Attachment of a palmitate lipid residue to the peptide can help peptides to cross the cell membrane. Various studies have used palmitoylation technology to study the functional receptors in platelets (Covic et al., 2002a, Edwards et al., 2007, Koloka et al., 2008, Stephens et al., 1998, Gkourogianni et al., 2013).

In the previous results chapters (3 and 4), the parent peptides; E-Cad 1, E-Cad 2 and N-Cad 2 and the described controls showed anti-platelet activity. In addition, the KEPLLP peptide and most of its controls also showed anti-platelet activity. Although most of the peptides inhibited the platelet activation in response to standard platelet agonist, this effect was not due to any overt toxicity. We concluded that much of this observed anti-platelet activity is non-specific in its nature. Therefore I queried whether the cause of the non-specific activity could be deduced from an analysis of the variation of the peptides features, for example solubility, positive amino acids (AAs) and hydrophobicity. Therefore, in this current chapter the different variables of peptides, which might influence their bioactivity, are explored.

The main objective of this study is to understand which variables may be responsible for the observed anti-platelet activity of peptides used in the previous chapters 3 and 4. In this chapter platelet responses from all peptides were considered. The potential for AA characteristics to affect the activity was

explored. The characteristics assessed were; overall positive charge, overall negative charge, solubility, hydrophobicity, the presence of a positive AA at first position and the total count of positive AAs at first three positions. This analysis may help in the design of palmitoylated peptides to target platelet function in future.

6.2 Results

6.2.1 Selection of peptides

Data from previous chapters suggested that some of the anti-platelet activity of palmitoylated peptides was non-specific. However, the reason for this anti-platelet activity is unknown. Therefore, peptides from previous chapters 3 and 4 were selected to analyze the different variables (e.g. solubility and positive charge AAs) that can associate with activity. In addition, peptides derived from another junctional protein known as occludin were also included. For this analysis, E-, N-, K- and VE-Cadherin peptides and their various controls were selected (Table 6.1). In addition, N-terminus deletion peptides derived from the E- and N-Cadherin peptides and KEPLLP control peptides were also selected. Peptides therefore varied in length from 6 - 21 residues long. C-terminal-deletion peptides of E-, N-Cadherin and alanine scanning peptides of KEPLLP were excluded from the study because of their ability to bias the sample. In addition, peptides with Ttds linker and peptides that are toxic in RBC lysis were also excluded in this analysis. In total 41 peptides were assessed of which 28 were inhibitory peptides (active) and 13 were non-inhibitory peptides (inactive) (Table 6.1). In this analysis, active peptides are the ones that can inhibit >40% inhibition of TRAP-induced platelet ATP

secretion at 50μM. Inactive peptide refers to peptides, which inhibit <40% of TRAP-induced platelet ATP secretion at 50μM. Peptide variables (Table 6.2) were defined in the following manner. Peptides that are soluble in water are considered as soluble peptides and peptides that are insoluble in water but soluble in 100% Dimethyl sulfoxide (DMSO), are considered as non-soluble peptides. For the count of the number of positive AAs, the numbers of Arginine (R) and Lysine (K) were assessed within each peptide. For the count of the number of negative AAs, Aspartic acid (D) and Glutamic acid (E) were considered as negative AAs. The percentage of hydrophobicity was calculated based on the number of hydrophobic residues (Alanine (A), Isoleucine (I), Leucine (L), Valine (V), Phenylalanine (F), Tryptophan (W) and Tyrosine (Y) in the total peptide. To observe the AA homogeneity within the selected (Table 6.1) peptides, the Weblogo method was used (Figure 6.1) (Crooks et al., 2004).

Table 6.1 List of cadherin-derived peptides selected from previous results (Chapters 3 and 4). Peptides were listed according to their percentage of inhibition of TRAP-induced platelet ATP secretion.

Peptide Acronym	Percentage of Inhibition	Peptide Sequence
Scramble of KEPLLP	100	PLPLEK
N-Cad 2	99	KRRDKERQAKQLLIDP
K-Cad 2	96	RRQRKKEPLIISKE
OCLN-2	92	KTRRKMDRYDK
Scramble of KEPLLP	90	EKPLLP
VE-Cad 2 Randomly scrambled	90	KKGLRSRAQHVRARR
VE-Cad 3 Randomly scrambled	90	REKIRQAQSELHRAKRHRLGPV
N- Cad 2 Randomly scrambled	89	LDKQRPDRIKKQDERL
K-Cad 1	88	KKEPLIISKE
E-Cad 1 5N	88	VKEPLLP
VE-Cad 3	88	RRRLRKQARAHGKSVPEIHEQL
VE-Cad 1 Randomly scrambled	87	RLQRARGRARHK
N- Cad 2 Scramnoncharged	87	RKKERDKILRPQALEQ
E-Cad 2 Reverse charge	86	EKPLLPPKKKT
E-Cad 1 3N	85	LLPPEDDT
E-Cad 2 Scramnoncharged	84	RDPTLLPDEEP
E-Cad 1 Randomly scrambled	84	PERVLARLKP
N- Cad 2 Di-reverse	84	DPLIQLAKRQKERDKR
E-Cad 1 2N	83	RAVVKEPLLP
E-Cad 1 Di-reverse	81	LPPLKEVVRARR
E-Cad 2	80	KEPLLPPEDDT
N-Cad 2 2N	77	RDKERQAKQLLIDP
OCLN-1	77	KTRRKMDRY
E-Cad 1	76	RRRAVVKEPLLP
N-Cad 1	74	KQLIIDPEDDV
E-Cad 1Scramnoncharged	71	KKKPLARDVVPL
E-Cad 2 5N	47	PPEDDT
N-Cad 2 6N	45	RQAKQLLIDP
N-Cad 2 3N	39	DKERQAKQLLIDP
E-Cad 2 6N	36	PEDDT
E-Cad 1 4N	29	VVKEPLLP
E-Cad 2 4N	28	LPPEDDT
E-Cad 2 Randomly scrambled	23	DPDLELTKPDE
N-Cad 2 5N	22	ERQAKQLLIDP
Scramble of KEPLLP	17	PLLPKE
E-Cad 1 Reverse charge	13	EEEAVVEKPLLP
E-Cad 2 Di-reverse	11	DTEDPPLLEPK
E-Cad 1 3N	9	AVVKEPLLP
N-Cad 2 7N	3	QAKQLLIDP
N-Cad 2 8N	1	AKQLLIDP
N-Cad 2 Reverse charge	1	EEEKEKEQAEQLLIKP

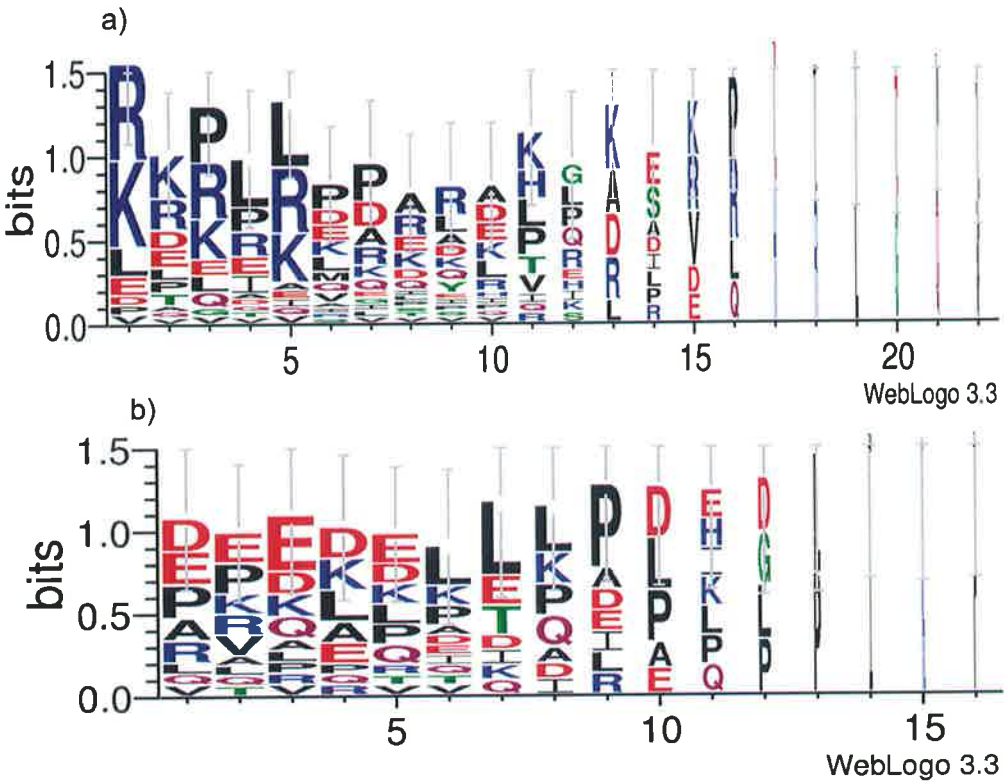


Figure 6.1 Graphical representation of amino acid homogeneity using web logo (http://weblogo.threeplusone.com/create.cgi). Weblogo of 28 active (a) and 13 inactive cadherin-derived peptides (b). Each logo consists of stacks of symbols, one stack for each position in the sequence. The overall height of the stack indicates the sequence homogeneity at that position, while the height of symbols within the stack indicates the relative frequency of each amino at that position. The width of the stack is proportional to the fraction of valid symbols in that position.

Table 6.2 List of different variables chosen for regression analysis form selected peptide sequences (Table 6.1) and their acronyms.

Name of the variable	Acronym
Solubility	Variable 1
Positive charge AA at first position	Variable 2
Count of positive charge AAs at first 3 positions	Variable 3
Number of positive charge AAs in the sequence	Variable 4
Number of negative charge AAs in the sequence	Variable 5
Percentage of hydrophobicity	Variable 6

6.2.2 Regression analysis of different variables of cadherin-derived peptides

To identify the effect of different peptide variables (Table 6.2) that can associate with activity, regression analysis was performed. For this analysis the adjusted R-squared value was chosen as a best estimator of association, since this measure can be calculated for both single predictor models and for multiple regression models with multiple predictors of platelet function inhibition. Adjusted R-squared represents the amount of variance in the predicted variable explained by the predictor variables and it is on a scale that ranges from zero to one. The regression output of single variable analysis predicted that activity of peptides is not associated with number of negative charges (variable 5) and hydrophobicity (variable 6) (Table 6.3). This single variable regression analysis also highlighted that solubility (variable 1) has a higher R-squared value (0.538) than either variable 3 (count of positive charge AAs at first 3 positions) or variable 4 (number of positive AA) (0.314 and 0.296, respectively) (Table 6.3). This suggests that water-soluble peptide is most likely to be active. Moreover, association of activity with any combination of different variables was also assessed. The combined analysis of two different variables suggested combination of variable 1 + variable 3 (solubility + count of positive charge AAs at first 3 positions and variable 1+ variable 2 (solubility + positive AA at first position) are associated with activity and their R squared values are 0.577 and 0.574 (Table 6.4). Other combinations variables were not strongly associated with activity (Table 6.4). Together, this regression analysis suggested that a water-soluble peptides

with a positively charge AAs at the first 3 positions has a higher probability of becoming an active peptide in the assays of platelet function.

Table 6.3 Pairwise correlation of activity association with individual variables of all peptides. P values refer to significance of estimate, where P<0.05, P<0.001 and P<0.0001.

Variable	R-Squared value	P value
Solubility (variable 1)	0.538	0.0000
Positive charge AA at first position (variable 2)	0.287	0.0001
Count of positive charge AAs at first 3 positions (variable 3)	0.314	0.0001
Number of positive charge AAs in the sequence (variable 4)	0.296	0.0000
Number of negative charge AAs in the sequence (variable 5)	0.007	0.261
Percentage of hydrophobicity (variable 6)	0.020	0.183

Table 6.4 Association of activity with combination two different variables. P values refer to significance of estimate, where P<0.05, P<0.001 and P<0.0001.

Variable	R-Squared value	P value
Solubility and positive charge AA at first position (variable 1 and 2)	0.574	0.0000
Solubility and positive charge AAs at first 3 positions (variable 1 and 3)	0.577	0.0000
Number of positive charge AAs in the sequence and positive charge AA at first position (variable 1 and 4)	0.399	0.0001
Count of positive charge AAs at first 3 positions and positive charge AA at first position (variable 3 and 4)	0.339	0.128
Count of positive charge AAs at first 3 positions and number of positive charge AAs in the sequence (variable 3 and 4)	0.350	0.086

6.2.3 Effect of positively charged amino acids on platelet function

Regression analysis predicted that water-soluble peptides with a positive (amino acid) AA at the first 3 positions of palmitoylated peptides could show inhibition of the platelet response to a standard agonist. Therefore, I next explored the effect of a simple positively charge AA attached to a palmitate moiety. To do this, I used three peptides: RRR, RR and R peptides. Peptides were synthesized with N-terminus palmitoylation and C-terminus amidation. The effect of Pal-RRR, Pal-RR and Pal-R was examined in the platelet ATP secretion assay. The ATP secretion assay identified that RRR, RR and R peptides significantly inhibited the TRAP induced platelet activation (Figure 6.2). In addition, Pal-R more significantly inhibited the TRAP induced platelet ATP secretion compared to Pal-RRR and Pal-RR ($P<0.0313$) (Figure 6.2). Moreover, Pal-RRR and RR showed some level of platelet activation in the absence of a platelet agonist (Figure 6.2). These results suggesting that positively charged palmitoylated peptides are probably sufficient to inhibit platelet secretion. In parallel, the toxicity of RRR, RR and R peptides was also analysed in RBC lysis assay at 50μM. Toxicity analysis showed that Pal-RRR and RR peptides are toxic to the RBC. Partial platelet activatory response of RRR and RR is probably due to the toxicity.

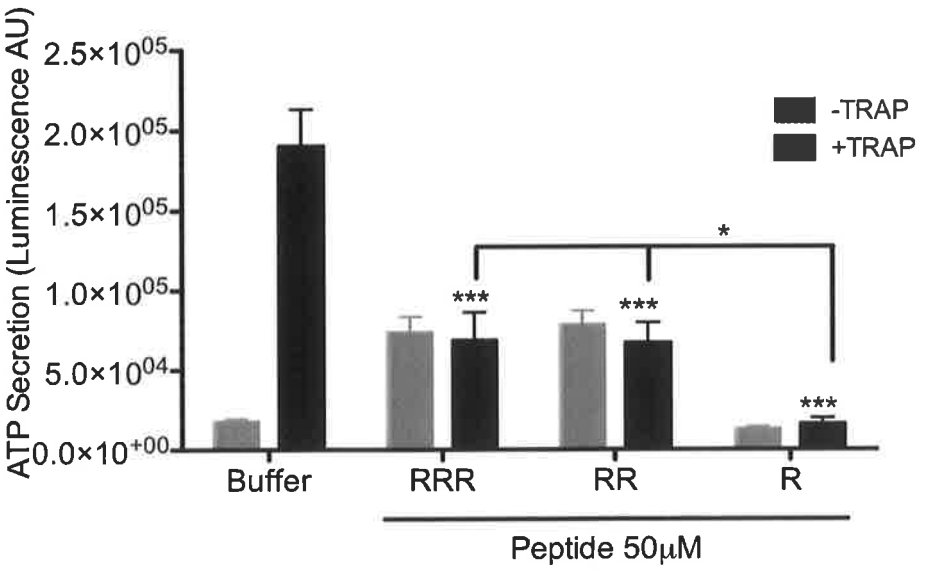


Figure 6.2 Palmitoylated RRR, RR and R peptides inhibit the platelet ATP secretion response to TRAP. Washed platelets were treated with 50μM of each peptide for 12 minutes at 37°C followed by activation of platelets with TRAP. Grey bars indicate the response of platelets to peptides in the absence of TRAP, while black bars indicate responses of platelets that have been activated with TRAP (4μM). Significance was compared with response of platelets to agonist in the absence of any peptides. Data shown is mean ±SEM of N=6 individual donors, where *P<0.05 and ***P<0.0001 represents statistical analysis calculated using a One-way ANOVA test.

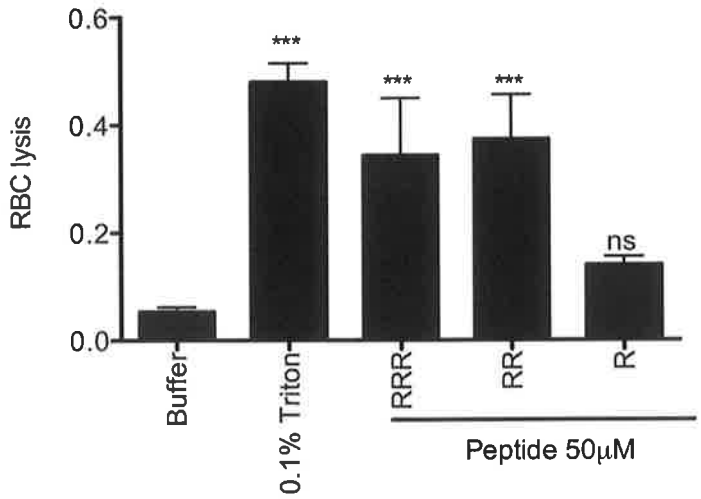


Figure 6.3 Toxicity analysis of Palmitoylated RRR, RR and R peptides in RBC lysis assay. Diluted RBCs (1 in 10) were treated with 50μM each peptide at 37°C for 15 minutes. Samples were incubated in a thermomixer at 300rpm. After incubation, samples were centrifuged at 1000xg for 5 minutes and the supernatant was collected and analysed at 405nm absorbance for presence of hemoglobin. Data represents mean ±SEM of N=3 individual expreiments. ***P<0.001 represent statistical significance values determined by One-way ANOVA. Significance was obtained by comparison of peptide or Triton-X-100 treated RBC with buffer treated RBC (negative control).

6.3 Discussion

The non-specific activity of palmitoylated peptides in platelets was never reported before. In this chapter the different variables of cadherin-derived peptides, which can show inhibitory activity on platelet function were investigated with a view to using the information to predict peptide activity. The regression analysis of different variables associated with activity suggested that peptides that contain palmitoylation modification, positive AAs in the first 3 positions with water-soluble property are crucial for inhibitory activity of platelet function. In addition, the prediction was further assessed using analysis of Pal-RRR, Pal-RR and Pal-R peptides.

Several studies have used palmitoylated peptides to understand the signaling rich portion of key receptors in platelets (Koloka et al., 2008, Covic et al., 2002a, Edwards et al., 2007). Parthasarathi *et al.* reported that absolute net charge can predict anti-platelet activity of peptides (Parthasarathi et al., 2006). Based on the peptide results from chapters 3 and 4, it was found that some palmitoylated peptides appeared to exhibit non-specific inhibition of platelet function. Understanding the antagonist activity of these peptides is critical. Therefore in this chapter, using regression analysis, it was predicted that the common variables among all active and inactive cadherin-derived peptides could facilitate an understanding of the nature of specific and non-specific peptide effects on platelet function. This study predicted two different variables, namely water-solubility and positive amino acid at first 3 positions are strong variables, which can inhibit the platelet secretion.

The analysis of Pal-RRR, Pal-RR and Pal-R peptides further suggested that soluble peptides with a positive charge adjacent to palmitoylation are responsible for the inhibition of platelet function. This may explain much of the non-specific effects observed in our earlier work. However, not all peptides that are water-soluble and that contain a positively charged amino acid at the first position are inhibitors of platelet secretion or platelet function. Stephens *et al.* demonstrated that Pal-KVGFFKR, derived from the juxtamembrane region of the major platelet integrin αIIbβ3, was a potent and specific platelet activator (Stephens et al., 1998). In those studies and in follow-on studies Bernard *et al.* demonstrated that a control peptide pal-KAAAAAR failed to have any impact on platelet aggregation, secretion or spreading (Bernard et al., 2009, Stephens et al., 1998). However, a related peptide Pal-KVGAAKR was a potent inhibitor of platelet function. Thus, while the presence of a positively charged AA next to N-terminal palmitoylation inhibited platelet secretion when combined with the cadherin-derived peptide sequence or variants, the presence of a positively charged amino acid (AA) is not the only determinant of an inhibitory response. This suggests that water solubility and positive AA at first 3 positions are not the only contributors to the overall activity of peptides. Other features of the cadherin-derived peptides must contribute to their inhibitory activity. However, the precise nature of any such features in the cadherin-derived peptides described in this thesis remains unknown. The potential for lipophilic nature of peptides to contribute to the observed responses was next explored. The rational was that highly lipophilic drugs like carvedilol (β-blocker) are also known to inhibit platelet function (Petrikova et al., 2002). They appear to exert this action by readily interfering

with structural components of cell membranes including phospholipids, enzymes and receptors (Petrikova et al., 2002). Another study showed that Midazolam, a sedative, could inhibit the platelet aggregation by causing changes in platelet membrane fluidity (Sheu et al., 2002). Alterations in membrane fluidity or conformational changes in the plasma membrane are generally acceptable mechanisms for anti-platelet activity of several drugs due to their lipophilic nature. The observed anti-platelet activity of the peptides used in this study may be associated with an alteration in the platelet membrane fluidity or interference with intracellular platelet activatory signaling molecules. An alteration in the platelet membrane fluidity in the presence of cadherin-derived peptides was attempted using fluorescent anisotropy to report on membrane fluidity.

Changes in the platelet membrane fluidity in the presence of cadherin-derived peptides were assessed using a membrane fluorescent dye; N,N,N-Trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)phenylammonium p-toluenesulfonate (TMADPH) (Sheu et al., 2002) in collaboration with laboratory based in Dublin City University. However, results did not explain the membrane fluidity even with the positive control. This effect is probably due to transportation of platelets from RCSI to DCU.

In future, designing of palmitoylated peptides should consider possible control peptide activity along with positively charged residues distribution within the peptide.

In conclusion, the analysis of various peptide variables in a small collection of 41 peptides derived from cadherin juxtamembrane sequences identified that water-soluble peptides with positively charged AAs adjacent to the palmitate moiety could significantly inhibit the platelet function. In future, this analysis may be useful to design more focused palmitoylated peptides to understand key receptors in platelet function.

Chapter 7

General Discussion

7.1 Discussion

Platelets are small anuclear blood cells derived from the fragmentation of precursor megakaryocytes. In addition to their role in hemostasis, platelets are also invloved in inflammation and the progression of cancer. Due to their lack of nucleus and genetic material, the capability of platelets have been underestimated in the past. However, various studies of platelet activity and function demostrate the significant role of platelets in various complex biological processes. The identifcation and understanding of novel platelet receptors and their contribution to platelet function will enhance our knowledge of various platelet related disease states. Such studies will help to develop novel therapecutic agents. The use of palmitoylation peptide technology is an ideal method to study receptor function, not only in platelets, but other cell types, for example endothelial cells.

Cadherins are single pass transmembrane cell adhesion molecules which are located at most cell-cell adherent junctions. They are involved in various physiological processes such as morphogenesis and embroyonic delopment, as well as pathophysiological processes such as cancer. It has been demonstrated that platelets express a number of cell junction adhesion molecules for example K-Cadherin, PECAM-1 and Junctional adhesion lolecule-A (JMA-A) on their surface. The understanding of the junctional proteins role in platelet function may aid the development of novel therapeutic agents to target platelet related diseases.

Edwards *et al.* identified a peptide derived from K-Cadherin that altered platelet function. Based on this obsevation, in chapter 3, I designed peptides from the JMD of K-, E- and N-Cadherins in order to gain a greater understanding of which peptide motif were able to inhibit platelet function most effectively. Peptides were synthasized with palmitoylation at the N-terminus and amidation at C-terminus (Edwards et al., 2007) in line with previous studies (Stephens et al., 1998, Bernard et al., 2009, Edwards et al., 2007). Assessment of the effect of cadherin-derived peptides on platelet function in response to standard platelet agonists revelead that E- and N-Cadherin peptides (parent peptides) also modulated the platelet function similar to K-Cadherin peptides from the Edwards *et al.* study. This suggests a potential role for cadherins in platelet function. To further explore the sequence specific activity of cadherin-derived parent peptides, control peptides were designed. In addition, key residues responsible for anti-platelet activity within parent peptides were identified, using a deletion study. This deletion analysis identified a short peptide squence (KEPLLP) as a potent inhibitor of platelet function. Based on this short motif, it was hypothesised that this KEPLLP peptide might show sequence specific activity. However, the analysis of E-Cad 1,2, N-Cad-2 control peptides and KEPLLP control peptides identified that the cadherin-derived peptides non-specifically inhibited platelet function. Several studies have used peptides as therapeutic drug targets. Attachment of palmitoylation or the TAT sequence will help the peptide to translocate across the cell membrane. For example KAI-9803 (SFNSYELGSL) peptide is developed from the δV1-1 region of protein kinase C (PKC) and is conjugated to TAT carrier peptide by a disulphide bond. KAI-

9803 was found to selectively inhibit PKC translocation and reduce the damage in cardiacmyocytes (Miyaji et al., 2011). This drug has been studied in clinical trials. Palmitoylated peptides derived from PAR1 and PAR4 inhibited the platelet-mediated thrombosis *in vivo* (Leger et al., 2006). This suggests that cell permeable peptides are useful to target various protein functions both *in vitro* and *in vivo*. Initial cadherin-derived peptide data was exiting. However, subsequent analysis of control peptides demonstrated that peptides can exhibit non-specfic inhibition of platelet function. Some of this non-specific activity can be explained by senstive toxicity assay (RBC lysis) but it cannot be entirely explained by toxicity analysis. This observation will help to design more specific anti-platelet peptides in the future.

According to the findings from chapter 3, I searched for the expression of different cadherins on human platelets. Dunne *et al.* have demonstrated that platelets express K-Cadherin on their surface (Dunne et al., 2012). Their search was prompted by McRedmond *et al.* who had shown evidence for K-Cadherin mRNA in platelets (McRedmond et al., 2004). In addition, Elrod *et al.* had demostrated the presence of E-Caderin in platelets (Elrod et al., 2003). In chapter 4, I therefore examined the expression of all potential cadherins on the platelet surface using theree seperate techniques incuding, western blots, flow cytometry and mass spectrometry. I demonstrated that there was no evidence for the presence E- or N-Cadherin in human platelets. This is in direct contrast to the report of Elord *et al.* (Elrod et al., 2003). However, the specific antibodies used in this study differed from those used in my study. I used different antibody (clone 24E10). In addition, I used a Pan-

Cadherin antibody which can detect multiple cadherins. All antibodies were verified through the use of known positive control cell lines. My study concluded that both K- and VE-Cadherin were present on human platelets. The observation of the presence of VE-Cadherin was entirely novel and needed to be verified. We used mass spectrometric techniques to verify the antibodies used in the western blots were specific and that peptide fragments corresponding to the VE-Cadherin sequence were present with higher confidence level in lysates prepared from human platelets. Peptide studies were performed using VE-Cadherin-derived SLIM motifs, exactly analogues to the motifs from E-,N- and K-Cadherins used in chapter 3 to investigate the role of VE-Cadherin in human platelets. However, for those peptides, I was careful to design suitable control peptides from each peptide sequence to be tested in the biological assays. The data from these studies confirmed that many of the effects observed appeared to be due to non-specific activity of the peptide sequences.

A number of attempts were then made to explore the role of VE-Cadherin in platelets. Platelet aggregation was not inhibited by blocking antibody against VE-Cadherin (BV9). Adhesion of platelets to immobilized VE-Cadherin and their spreading similar to fibrinogen suggested a role for VE-Cadherin. This adhesion could be due to the RGD motif in the second extracellular domain of VE-Cadherin. The role of RGD in VE-Cadherin was explored by platelet adhesion to immobilised VE-Cadherin in the presence of RGD and Abciximab (integrin αIIbβ3 antagonist). In the presence of RGD and Abciximab platelet adhesion to VE-Cadherin was significantly inhibited. This result suggested

that VE-Cadherin can bind to platelet integrin αIIbβ3 in a heterophilic manner. However, it is not clear from these studies whether the interaction is direct or indirect. Previously, it has been shown that VE-Cadherin can form an heterophilic interaction with the common platelet integrin αIIbβ3 ligand, fibrinogen (Yakovlev and Medved, 2009). Fibrinogen/VE-Cadherin interaction is mediated by the β-chain (15-66) of fibrinogen and the third extracellular domain of VE-Cadherin (Yakovlev et al., 2011). It is possible that VE-Cadherin could mediate an interaction with platelets via fibrinogen binding. Thus fibrinogen, possibly released from α-granules in activated platelets, could act as a bridge linking between VE-Cadherin and platelets. RGD and abciximab would be expected to inhibit this interaction by inhibiting fibrinogen binding to the platelet integrin. However, using CHO cells which express integrin αIIbβ3, I demonstrate that the interaction between the integrin and VE-Cadherin must be direct since there is no possible source of fibrinogen in the experiments that utilized CHO cell lines expressing αIIbβ3; yet the CHO cells expressing the integrin adhere to immobilized VE-Cadherin, in contrast to mock transfected cells which do not express integrin.

Based on the observations in chapter 4, the role of VE-Cadherin could be hypothesized in two different ways. At the damaged vessel, VE-Cadherin on platelets could form interactions with VE-Cadherin on endothelial cells. Alternatively, endothelial integrins such as αvβ3 could form adhesive interactions with platelet VE-Cadherin. Rosenblum *et al.* suggested that PECAM on platelets plays a role in platelet adhesion/aggregation at minor vascular injuries where there is no exposure of extracellular matrix (ECM)

(Rosenblum et al., 1996). However, their study did not confirm whether PECAM on endothelial cells or PECAM on platelets is responsible for platelet binding to minor vascular injuries. Based on these observations I propose that, similar to PECAM, VE-Cadherin might play a role in platelet adhesion/aggregation at sites of minor injuries where there is no exposure of ECM.

VE-Cadherin is important in a number of physiological and pathophysiological processes, for example, angiogenesis. Angiogenesis is crucial for morphogenesis and organ development, but it also plays an important role in the progression of cancer. Agents that can inhibit tumour associated angiogenesis could be useful therapeutic agents for the treatment of cancer. Previous studies have shown that anti-angiogenic peptides were designed from various proteins such as extracellular matrix proteins, growth factors receptors, coagulation cascade proteins and chemokines (Rosca et al., 2011). The effect of some of these peptides were studied in both *in vitro* and *in vivo* (Rosca et al., 2011). In chapter 5, I used peptides derived form VE-Cadherin. The effect of VE-Cadherin peptides was assessed in various assays of endothelial cell function. At this stage in my thesis, I was interested to know if any of the peptides designed and described in the earlier chapters might show some aspect of selective function in assays that were well-established to be dependent on cadherin function. VE-Cadherin is the most abundant adhesion molecule on vascular endothelial cells. VE-Cadherin peptides specifically and significantly inhibited endothelial capillary tube formation, endothelial cell migration, and cell proliferation. These observations were made using

carefully controlled pairs of query peptides and matched control peptides. In addition, the doses of the peptides used were lower than had been used in the previous chapters. The striking ability of some of the peptides (notably VE-Cad 2 and VE-Cad 4 peptides) to selectively inhibit endothelial function in these 3 assays suggest potential role for these peptides as therapeutic anti-angiogenic agents. In addition, these peptides demonstrate a key role for membrane proximal region of VE-Cadherin in endothelial cell function.

The final section of this thesis investigated the link between inhibition of platelet function and different variables of the palmitoylated peptides. Based on experimental data from chapter 3 and 4, regression analysis of different peptide variables association with activity predicted that water soluble peptides with positively charged AAs at their N-terminus are crucial for inhibition of platelet function. In addition, I experimatally verifed this regression analysis prediction using positively charged palmitoylated peptides, Pal- RRR, Pal-RR and Pal-R. These results suggested that inhibition of platelet function using palmitoylated peptides was probably associated with positively charged AAs at the N-terminus of the peptides. However, further investigation is required to identify what combinations of AAs attached to palmitoylation can inhibit platelet function and to understand the impact of these peptides on platelet function.

In conclusion, this thesis has highlighted the potential for non-specific effects of palmitoylated cadherin-derived peptides not previously observed in the literature. It has provided some structural understanding of the peptide

features that may be causative for these non-specific actions and should enable more detailed and productive analysis of peptides to be performed in the future. In addition, this thesis has furthered scientific knowledge of VE-Cadherin in human platelets. It has demonstrated that VE-Cadherin is a novel direct ligand of platelet integrin αIIbβ3 via an RGD dependent mechanism. The impact of exclusive RGD dependent binding of VE-Cadherin-αIIbβ3 interaction could have a potential impact on the use of RGD derived drugs for the treatment of thrombosis. RGD-based drugs such as eptifibatide and Tirofiban are used in the treatment of acute thrombotic events as they prevent platelet-platelet adhesion via fibrinogen. However, if they also inhibit potential platelet-endothelial interactions, their usefulness in patient treatment could be expanded. In addition, this thesis has demonstrated the evolution of a peptide design process that allowed the identification of cadherin-derived peptides that demonstrated selective inhibition of VE-Cadherin function in vascular endothelial cells. This approach may facilitate the identification of peptide inhibitors of other biological responses in the future. In particular, VE-Cad 2 and 4 peptides show a potent ability of to target key functions in angiogenesis. These peptides could be useful tools or template drugs for the development of novel anti-angiogenic approaches to inhibit angiogenesis in pathological conditions such as cancer by targeting the various proteins involved in angiogenesis.

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